Title Page

LONG CHAIN N-3 POLYUNSATURATED FATTY ACID INHIBITION OF MAST CELL ACTIVATION

by

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ABSTRACT

Allergic diseases are a major medical concern with increasing prevalence in the world. Inflammation is one of the hallmarks of allergic diseases. Long chain n-3 polyunsaturated fatty acids (PUFAs) have been shown to inhibit allergic inflammation, but their direct effect on activation of the mast cell, a major effector cell in allergic inflammation, is poorly understood. We hypothesized that long chain n-3 PUFAs inhibit mast cell activation through alteration of the expression of some signaling molecules and modification of Fc ε receptor I (FcεRI) association with lipid rafts, membrane microdomains containing high levels of cholesterol, sphingolipid, transmembrane proteins, acylated proteins, and glycosylphosphatidylinisotol (GPI)-anchored proteins. Bone marrow-derived mast cells (BMMC) were cultivated from C57BL/6 wild type (WT) and fat-1 transgenic mice which express fatty acid n-3 desaturase and produce endogenous n-3 PUFAs. Exogenously, long chain n-3 PUFAs were supplemented to BMMC and human mast cell line laboratory of allergic diseases 2 (LAD2) cells. Mast cell degranulation, lipid-derived mediator release, and cytokine/chemokine production were evaluated following cell activation through FcεRI. FcεRI expression and signal transduction was determined by flow cytometry and western blot analysis.

GC-MS analysis showed increased long chain n-3 PUFAs levels in whole cell and lipid rafts of fat-1 BMMC, compared to WT, and in whole cell long chain n-3 PUFA-treated BMMC. Fat-1 BMMC produced less tumor necrosis factor (TNF) and CC chemokine ligand 2 (CCL2), and cysteinyl leukotrienes (cys-LTs), and showed reduced degranulation compared to WT BMMC after cell stimulation with immunoglobulin E (IgE)/antigen. Long chain n-3 PUFA exogenous supplementation caused decreased

degranulation, cys-LT synthesis and cytokine/chemokine production in activated BMMC and LAD2 cells. Western blot analysis showed that the expression of Lyn and linker for activation of T cells (LAT) and phosphorylation of Lyn and spleen tyrosine kinase (Syk) and LAT was inhibited in fat-1 BMMC compared to WT. In both BMMC and LAD2 cells, long chain n-3 PUFAs did not alter cell surface and whole cell FcεRI expression. However, FcεRI was excluded from lipid rafts by long chain n-3 PUFAs in resting mast cells. Furthermore, the shuttling of FcεRI to rafts of activated mast cells was inhibited by long chain n-3 PUFAs. After lipid raft disruption by depleting cholesterol, mast cell degranulation, cys-LT release and cytokine/chemokine production were reduced in both WT and fat-1 BMMC. In addition, FcεRI and Lyn localization to lipid rafts were suppressed by MBCD treatment. This study clearly indicates that long chain n-3 PUFAs inhibit FcεRI-mediated signal transduction and mediator release from mast cells by disrupting FcεRI localization and partitioning to lipid rafts, and supressing the expression of Lyn and LAT. Cholesterol is required for the inhibitory effect of long chain n-3 PUFAs on mast cell activation.

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This period of time is meant to be unforgettable and fateful in my life. With all unforeseen difficulties, good and bad lucks, and extremely valuable knowledge I learned, I feel like I am a different person.

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List of Abbreviations

AA	Arachidonic acid
ALA	α-Linolenic acid
ANOVA	Analysis of variance
AP-1	Activator protein-1
β-hex	β-hexosaminidase
BAD	B cell lymphoma-2-associated death promoter
BALF	Bronchoalveolar lavage fluid
BCR	B cell receptor
BMMC	Bone marrow-derived mast cells
BTK	Bruton's tyrosine kinase
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
COX	Cyclooxygenases
cPLA2	Cytosolic phospholipase A2
CTMC	Connective tissue-type
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
Cys-LT	Cysteinyl leukotriene
CysLTR	Cysteinyl leukotriene receptor
DAG	Diacylglycerol

DGLA	Dihomo-γ-Linolenic acid
DHA	Docosahexaenoic acid
DHC	7-dehydrocholesterol
DHCR	3β-hydroxysterol Δ7-reductase
DMSO	Dimethyl sulfoxide
DNP	2,4-dinitrophenyl
DPA	Docosapentaenoic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FAME	Fatty acid methyl ester
FcεRI	Fc ε receptor I
FLAP	5-lipoxygenase activating protein
GAB2	Growth factor receptor-bound protein 2 (Grb2)-associated- binding protein 2
GC-MS	Gas chromatography-mass spectrometry
GM1	Monosialotetrahexosylganglioside
GPI	Glycosylphosphatidylinositol
GPR	G-protein-coupled receptor
Grb2	Growth factor receptor-bound protein 2

GM-CSF	Granulocyte/macrophage-colony stimulating factor
HSA	Human serum albumin
HER-2	Human epidermal growth factor receptor-2
HR	Histamine receptor
HRP	Horseradish peroxidase
hr	Hour (s)
ICAM	Intercellular adhesion molecule
IFN-γ	Interferon-γ
IFN-γR	Interferon-γ receptor
IgE	Immunoglobulin E
IL	Interleukin
IL-18BP	IL-18 binding protein
IL-2R	IL-2 receptor
IP3	Inositol trisphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
JNK	c-Jun N-terminal kinase
LAD2	Laboratory of allergic diseases 2
LAT	Linker of Activated T cells
LFA	Lymphocyte function-associated antigen
LOX	Lipoxygenases
LPS	Lipopolysaccharide
LT	Leukotriene

MAPK	Mitogen-activated protein kinase
MBCD	Methyl-β-cyclodextrin
MCT	Mast cell containing tryptase
MCTC	Mast cell containing tryptase and chymase
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
min	Minute (s)
MMC	Mucosal mast cells
mTOR	Mammalian target of rapamycin
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NK	Natural killer
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
PAF	Platelet-activating factor
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PDK	Phosphoinositide-dependent kinase
PE	Phycoerythrin
PG	Prostaglandin
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate

PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PLC-γ	Phospholipase C-γ
PLD	Phospholipase D
PPAR	Peroxisome proliferator-activated receptor
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
PUFA	Polyunsaturated fatty acid
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SCF	Stem cell factor
SEM	Standard error of the mean
SH2	Sarcoma homology 2
SHIP	SH2-containing Inositol 5-Phosphatase
SHP-1	SH2-containing phosphatase-1
SLP76	SH2 domain containing leukocyte protein of 76kDa
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SOS	Son-of-sevenless homologue (SOS)
Src	Sarcoma
STAT	Signal transducer and activator of transcription
Syk	Spleen tyrosine kinase

TBS	Tris-buffered saline
TCR	T cell receptor
TGF-β	Transforming growth factor-β
Th	T helper
TLR	Toll-like receptor
TNE	Tris/NaCl/EDTA
TNF	Tumor necrosis factor
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WT	Wild type
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Mast cells and their role in inflammation and disease

1.1.1 A brief introduction of mast cells: properties, origin and types

Mast cells are tissue residing cells found throughout the body, preferentially in tissues that have direct interaction with the outer environment, such as skin, airway and gastrointestinal tracts (Abraham and St John, 2010). It has been suggested that mast cells would be as large as a human spleen if gathered in a single organ (Sayed et al., 2008). Mast cells can be found in all vertebrates, and are believed to be ancient immune cells that are a part of the innate immune defense system (Silver and Curley, 2013). In their cytoplasm, mast cells contain many granules that are rich in histamine, heparin, neutral proteases, acid hydrolases, major basic protein, carboxypeptidases, and some preformed cytokines and growth factors (Palker et al., 2010). The granule contents differ in mast cells residing in different tissues (Miller and Pemberton, 2002). Mast cell granules stain positive with toluidine blue because the granules contain negatively charged heparin-proteoglycan-protease complexes (Kitamura, 1989).

Mast cells originate from pluripotent hematopoietic stem cells in bone marrow (Kirshenbaum et al., 1991). Immature mast cells leave the bone marrow, enter circulation, and then complete their differentiation in the local environment of the peripheral tissues in which they reside (Kambe et al., 2004). Mature mast cells are not present in the circulation under physiological conditions (Kitamura and Fujita, 1989). Both murine and human mast cells require stem cell factor (SCF), the ligand for cluster of differentiation (CD)117/c-Kit, for survival and proliferation (Galli et al., 1995). Interleukin (IL)-3 is critical for the in vitro cultivation of murine mast cells from their bone marrow precursors (Lantz et al., 1998). Other cytokines and growth factors may

also contribute to the development of mast cells, including IL-4, IL-9, IL-10, transforming growth factor beta (TGF-β) and nerve growth factor (NGF) (Okayama and Kawakami, 2006). On their own, these factors do not have direct effects on mast cell survival, proliferation, and response to activator, but they act synergistically with SCF or IL-3. For example, IL-4 does not support mast cell survival by itself, but it enhances mature mast cell proliferation and mediator release together with SCF by facilitating activation of mitogen-activated protein kinase (MAPK) and Akt pathways (Feuser et al., 2011). IL-4 also promotes mature mast cells to produce T helper 2 (Th2) cytokines such as IL-5 and IL-13, and reduce tumor necrosis factor (TNF) and IL-6 synthesis (Lorentz et al., 2000). Interestingly, IL-4 has different effects on immature mast cells. It was reported that IL-4 decreases mast cell numbers in peripheral blood $CD34^+$ progenitor cultures (Kulka and Metcalfe, 2005).

Two types of rodent mast cells, connective tissue-type mast cells (CTMC) and mucosal-type mast cells (MMC), were documented based on different morphological and histochemical characteristics, and tissue homing preference (Enerback, 1966a). CTMC and MMC have different proteoglycan content in granules. Heparin content is high in CTMC, but lacking in MMC. MMC granules contain chondroitin sulphates A and B, which are not present in CTMC. Chondroitin sulphate E was found in granules of both types of mast cells (Enerback, 1966c). After alcian blue/safranin staining sequence, granules of MMC stain blue with alcian blue and granules of CTMC stain red with safranin. MMC and CTMC also express different profiles of proteases and membrane molecules, so they have different responses to stimulators and have different functions (Enerback, 1966b). Two types of mast cells have also been discovered in human, those

containing tryptase (MCT) and those containing tryptase and chymase (MCTC). MCTC also contain cathepsin-G, and carboxypeptidase, and are found mainly in connective tissues, such as skin, submucosa of stomach and intestine, breast parenchyma, and lymph nodes. MCTC seem to be similar to CTMC in rodents. MCT lack the other neutral proteases seen in MCTC and are located predominantly in the mucosa of the respiratory and intestinal tracts. MCT are believed to correspond closely to rodent MMC (Irani and Schwartz, 1994).

1.1.2 Mast cell activation and products

Mast cells can be activated by many different types of stimuli, including allergens, complement, antimicrobial peptides, adenosine, neuropeptides and physical stimuli such as low temperature (Galli et al., 2005). The principle activation pathway is mediated by the high affinity immunoglobulin E (IgE) receptor, Fc ε receptor I (FcεRI). In the 1970s, IgE was found to induce the release of histamine from mast cells (Ishizaka and Ishizaka, 1978). FcεRI was discovered later and completely cloned in 1989 (Blank et al., 1989). FcεRI is expressed on mast cells as a tetrameric αβγ2, which mediates mast cell's interaction with IgE (Paolini et al., 1991). The cross-linking of IgE-bound FcεRI by bivalent or multivalent antigen results in the initiation of receptor signaling, subsequent signal transduction, and mast cell activation. FcεRI will be discussed in more detail in section 1.2. After activation, generally three groups of mediators are gradually released from mast cells. First, preformed mediators in granules, such as histamine and heparin, are released in seconds in a process termed degranulation. The second group of mediators is lipid-derived mediators such as prostaglandins (PGs) and leukotrienes (LTs), which are released in minutes. The third group of mediators is cytokines,

chemokines and growth factors which are released after gene transcription (Table 1.1). Mast cells can also produce other molecules such as nitric oxide (NO), reactive oxygen species (ROS) and antimicrobial peptides (Kalesnikoff and Galli, 2008). The first two groups are responsible for acute-phase reactions in allergic diseases resulting in vasodilation and increased vascular permeability (Hofmann and Abraham, 2009). The late-phase reactions, such as tissue swelling, fibrin deposition and leukocyte accumulation, are more often attributed to cytokines and chemokines (Hofmann and Abraham, 2009). By releasing mediators, mast cells play an important role in both the innate and adaptive immune responses and are especially vital in IgE-mediated allergic inflammation (Stelekati et al., 2007).

Table 1.1. Mediators produced by activated mast cells

Mediators	Species	References
Preformed mediators		
Histamine	Human, mouse	(McClain et al., 1983; Wang and Lau, 2007)
Serotonin	Human, mouse	(Kushnir-Sukhov et al., 2007; Ringvall et al., 2008)
Heparin	Human, mouse	(Craig et al., 1993; Lindstedt et al., 1992)
Tryptase	Human, mouse	(Schwartz, 1990; Valchanov and Proctor, 1999)
Chymase	Human, mouse	(Brown et al., 2003; Craig and Schwartz, 1989)
Major basic protein	Human	(Butterfield et al., 1990)
Cathepsin	Human	(Meier et al., 1985)
Carboxypeptidase A	Human, mouse	(Goldstein et al., 1989; Serafin et al., 1987)
Lipid-derived mediators		
PGD_2	Human, mouse, rat	(Lewis et al., 1982; Noguchi et al., 2005)
PGE_2	Rat, mouse	(Marshall et al., 1999)
LTB_4	Human, mouse	(Freeland et al., 1988; Miyahara et al., 2009)
LTC_4 and D_4	Human, mouse	(Bartosz et al., 1998; Razin et al., 1982)
PAF	Mouse	(Mencia-Huerta et al., 1983)
Cytokines/chemokines/growth factors		
TNF	Human, mouse	(Hochdorfer et al., 2013; Oksaharju et al., 2009)
IL-1β	Human, mouse	(Guma et al., 2010; Zhang et al., 2012a)
IL-4	Human, mouse	(Bradding et al., 1992; Sherman et al., 1999)
IL-5	Human, mouse	(Chiba et al., 2007; Lorentz et al., 1999)
IL-6	Human, mouse	(Hochdorfer et al., 2013; Kandere-Grzybowska et al., 2006)
IL-8	Human, mouse	(Feoktistov and Biaggioni, 1995; Oksaharju et al., 2009)
IL-10	Human, mouse	(Chiba et al., 2007; Ishizuka et al., 1999)
IL-13	Human, mouse	(Chiba et al., 2007; Toru et al., 1998)
IL-17	Human	(Lin et al., 2011)
IL-33	Mouse	(Hsu et al., 2010)

IFN-γ	Rat	(Gupta et al., 1996)
TGF-β	Human, mouse	(Kanbe et al., 1999)
CCL11 (eotaxin)	Mouse	(Hogaboam et al., 1998)
CCL2	Human, mouse	(Toda et al., 2012; Venkatesha et al., 2005)
CCL5	Human	(Venkatesha et al., 2005)
CXCL1/ CXCL2	Mouse	(De Filippo et al., 2013)
SCF	Human	(Zhang et al., 1998)
VEGF	Human, mouse	(Jimenez-Andrade et al., 2013; Sismanopoulos et al., 2012)
NGF	Human, rat, mouse	(Leon et al., 1994; Nilsson et al., 1997; Xiang and Nilsson, 2000)
PDGF	Human	(van Steensel et al., 2012)
Free radicals		
ROS	Human, rat, mouse	(Swindle et al., 2004)
Others		
Urocortin	Human	(Kempuraj et al., 2004)

IL: Interleukin; PG: Prostaglandin; LT: Leukotriene; PAF: Platelet activating factor; TNF: Tumor necrosis factor; IFN: Interferon; TGF: Transforming growth factor; CCL: CC chemokine ligand; CXCL: C-X-C chemokine ligand; SCF: Stem cell factor; VEGF: Vascular endothelial growth factor; NGF: Nerve growth factor; PDGF: Platelet-derived growth factor; ROS: Reactive oxygen species.

1.1.3 Role of mast cells in inflammatory diseases

Mast cells are considered important effector cells in certain types of inflammation (Theoharides et al., 2012). Mast cells are especially vital in IgE-mediated immediate type I hypersensitivity because of their quick and massive release of preformed mediators, lipid-derived mediators and cytokine/chemokines (Theoharides and Kalogeromitros, 2006). The classical viewpoint is that mast cells are important effector cells in allergic responses, such as allergic asthma. However, mast cells also play a role in other inflammatory diseases whose pathogenesis does not involve IgE (Bot et al., 2008).

Allergic asthma is a common IgE-mediated chronic inflammatory disease of the airways, characterized by airway inflammation, bronchial hyperreactivity, and airway remodelling (Barnes, 2008). Mast cells may participate in all of these processes. Angiogenesis promoting factors, such as vascular endothelial growth factor (VEGF), from activated mast cells contribute to airway remodelling in asthma (Zanini et al., 2007), which includes immune cell infiltration, mucus gland hypertrophy, and hyperplasia of epithelial cells and airway smooth muscle cells (Yuksel et al., 2013). Mast cells produce CC chemokine ligand (CCL)11 and histamine to attract eosinophils, another vital effector cell in allergic asthma responsible for both airway damage and remodelling (Shakoory et al., 2004). Histamine from mast cells causes smooth muscle contraction and may also promote mucus production by goblets cells (Huang et al., 2013; Suzuki and Kou, 1983). Mast cells also contribute to airway smooth muscle contraction by producing PGs and LTs (Krell et al., 1981; Tamaoki et al., 1987).

Another contribution of mast cells to the development of allergic asthma is their connection to Th2 cell responses (Nagarkar et al., 2012) which are more involved in late phase reactions. Mast cells produce IL-4, IL-5, and IL-13 (Ho et al., 2007; Sewell et al., 1998), which are among the most well characterized asthma-promoting Th2 cytokines. IL-4 plays a key role in initiation and amplification of Th2 responses and asthmatic reactions. IL-4 directs the polarization of naive helper T cells toward the Th2 subset by facilitating the expression of GATA-3, the crucial transcription factor for Th2 differentiation (Seki et al., 2004). In addition, IL-4 promotes the production of IgE from B cells (Pene et al., 1988), which mediates the activation of mast cells through interaction with FcεRI. IL-5 stimulates the production and terminal differentiation of eosinophils in the bone marrow and primes eosinophils to increase functional responses to lipid mediators, complements, and chemokines (Kuo et al., 2001; Takafuji et al., 1991). Furthermore, IL-5 is required for the release of eosinophils from bone marrow to the peripheral blood (Wang et al., 1998). IL-13 promotes eosinophil production and differentiation by increasing local IL-5 and eosinophil-attracting CCL11 expression (Gessner et al., 2005). Furthermore, IL-13 directly promotes airway smooth muscle cell contractility (Farghaly et al., 2008). IL-4 and IL-13 were also reported to facilitate IgG1 production (Lai and Mosmann, 1999) which may complex with inhaled antigen and activate dendritic cells through the Fcγ receptor to promote allergic responses (Ishikawa et al., 2011).

Mast cells also have a role in the development of inflammatory diseases that are not mediated by IgE. For example, heart mast cells, which reside predominantly in the shoulder region of human coronary atheroma, especially around nerve endings

(Kaartinen et al., 1994), are involved in the development of atherosclerosis, coronary inflammation, and cardiac ischemia (Bot et al., 2008). Mast cell proteases have been suggested to participate in neuroinflammation by activating microglia to produce TNF, IL-6, and ROS (Zhang et al., 2012b).

1.2 FcεRI and mast cell activation

1.2.1 FcεRI structure and regulation of FcεRI cell surface expression

FcεRI is a multimeric cell-surface receptor to which the Fc region of IgE binds with high affinity. FcεRI exists as two forms, trimeric and tetrameric molecules. Trimeric FcεRI is formed from one α and two γ chains, with no β chains present, referred to as αγ2. Trimeric FcεRI is expressed on human antigen-presenting cells, including macrophages, myeloid dendritic cells, plasmacytoid dendritic cells, Langerhans cells, and eosinophils, but is not present on rodent cells (Kinet, 1999).The tetrameric form of FcεRI contains one α-chain, one β-chain, and a homodimer of γ-chains linked by disulphide bonds, referred to as αβγ2 (Fig. 1.1). The tetrameric form of FcεRI exists on both human and rodent mast cells and basophils (Kraft and Kinet, 2007).

The α chain of FcεRI belongs to the immunoglobulin super family, which contains two extracellular immunoglobulin-related domains for IgE binding. FcεRIα also has a transmembrane domain and a short cytoplasmic tail with no kinase activity and no immunoreceptor tyrosine-based activation motif (ITAM) domain (Fig. 1.1) (Kimura et al., 1996). The α-chain contains seven N-glycosylated sites required for proper folding in the endoplasmic reticulum (ER), transportation from ER to the Golgi complex, and cell surface expression of FcεRI (Letourneur et al., 1995).

The β chain of FcεRI contains four transmembrane domains and two cytoplasmic tails. One of the tails has an ITAM sequence which functions as a tyrosine phosphorylation site. The γ chain has a short extracellular domain, a transmembrane domain, and an ITAM-containing cytoplasmic tail. Phosphorylated ITAMs in β and γ chains recruit and activate the sarcoma (Src) homology 2 (SH2) domain containing intracellular signaling molecules such as spleen tyrosine kinase (Syk). The γ chain is also required for expression and proper function of cell surface FcεRI because the γ chain releases the α chain from the ER by masking an ER-retention motif in the α chain (Fig. 1.1) (Kimura et al., 1996).

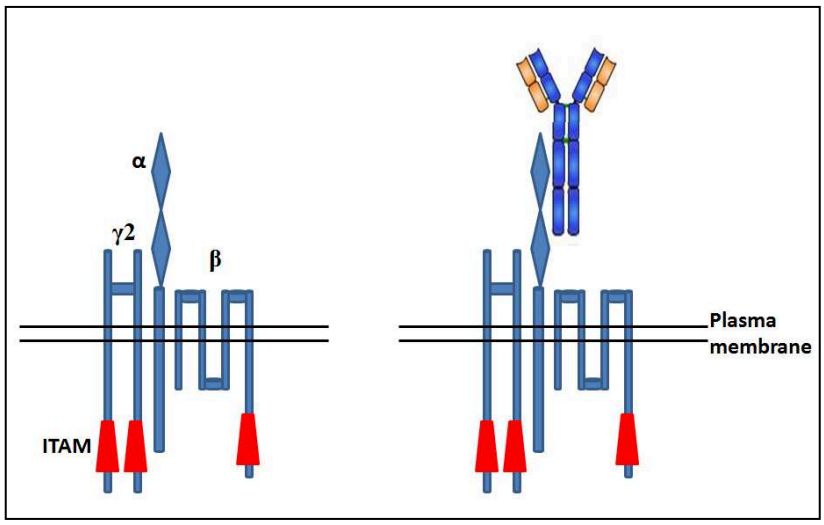

Figure 1.1 Structure of tetrameric FcεRI and binding of IgE to FcεRI. A tetrameric FcεRI is composed of one α chain, one β chain and two γ chains. β and γ chains contain ITAM in cytoplasmic tails for tyrosine phosphorylation. IgE binds to the α chain of FcεRI through constant Cε3 region. IgE: imunoglobulin E; ITAM: immunoreceptor tyrosine-based activation motif.

The sequence of events leading to FcεRI cell surface expression can be described as follows: The α chain is produced in the ER in an immature glycosylated form which stays in the ER in the absence of other FcεRI chains. After proper folding in the ER and assembly of the α, β and γ chains (or α and γ chains), immature FcεRI leaves the ER and enters the Golgi complex for terminal glycosylation. Mature FcεRI is then transported to the cell surface by secretary vesicles. The cell surface FcεRI are routinely internalized back to endosomes for degradation in lysosomes (Kraft and Kinet, 2007).

The cell surface expression of FcεRI can be regulated by several factors (Fig. 1.2). The β-chain, which has four transmembrane domains, favors the maturation and stability of the receptor complex, so the β-chain may function as an amplifier of cell surface FcεRI expression. It is reported that the cell surface expression of FcεRI in αγ2 expressing cells was significantly enhanced by introduction of FcεRIβ (Lin et al., 1996). A truncated β-chain variant which does not have a normal transmembrane domain and carboxyl terminus, βT, can be produced by alternative splicing. The incorporation of βT with an α chain results in degradation of immature FcεRI complexes in proteasomes (Donnadieu et al., 2003). Introduction of βT into αβγ2-expressing mast cells results in a significant decrease in FcεRI cell surface expression (Ra et al., 2012). Another modulator on FcεRI cell surface expression is FcεRI ligand, IgE. Monomeric IgE was shown to increase cell surface expression of FcεRI by stabilizing the receptor and reducing internalization (Quarto et al., 1985; Yamaguchi et al., 1997).

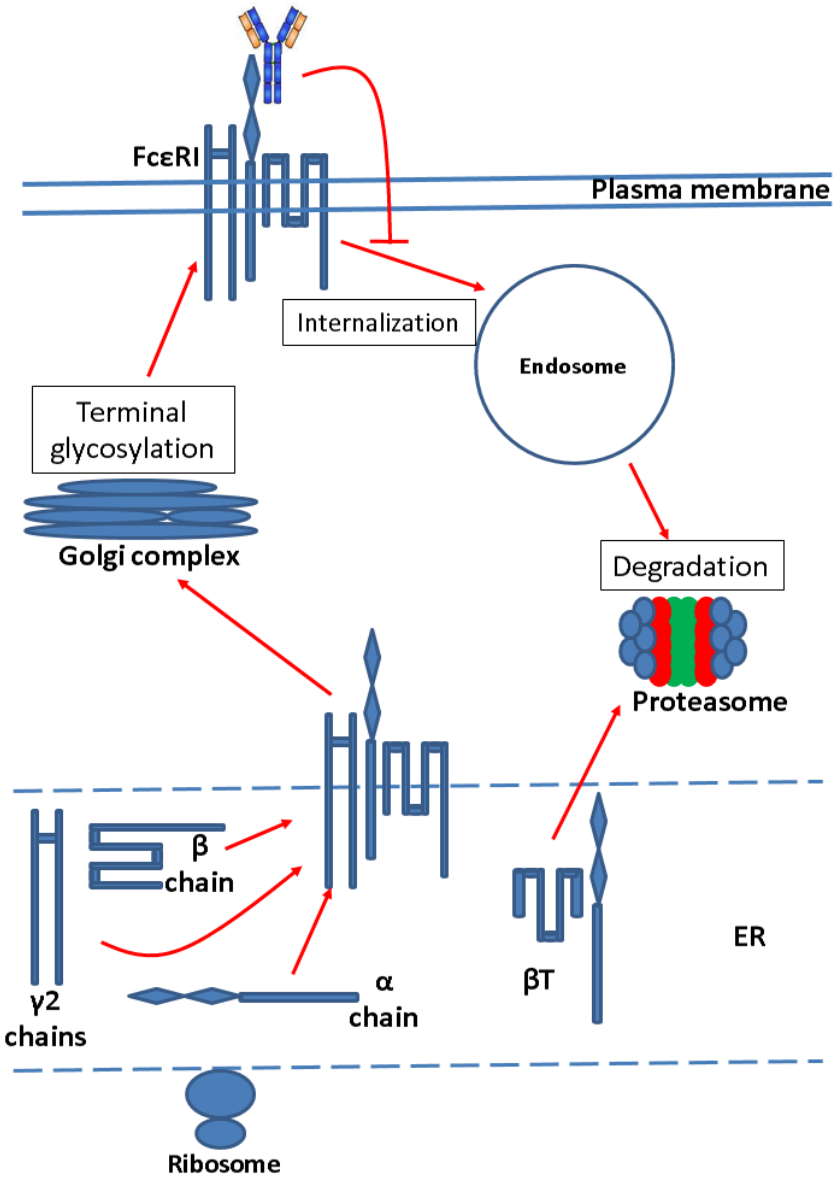

Figure 1.2 Regulation of FcεRI cell surface expression. α, β and γ chains are produced in the ER and assembled into tetrameric form, which is then transported to the Golgi complex for terminal glycosylation. After that, FcεRI is transported to the cell surface. A truncated version of the β chain, βT, can be produced in the ER by alternative splicing. The complex of α and βT will be degraded in the proteasome. Cell surface FcεRI is constantly internalized in the endosome and transported to the proteasome for degradation. The binding of IgE to FcεRI can suppress the receptor internalization. ER: endoplasmic reticulum; βT: truncated β chain.

1.2.2 Biological functions of tetrameric FcεRI

The most documented function of tetrameric FcεRI is as the key participant in IgE-mediated mast cell activation and allergic reaction (Ishizuka et al., 1996). Upon cross-linking of IgE-bound FcεRI by bivalent or multivalent antigens, mast cells are activated to gradually release preformed mediators, lipid-derived mediators, and cytokines/chemokines, which mediate early and late-phase allergic reactions (Liu et al., 2005). The level of FcεRI expression on the cell surface determines the mast cell's response to IgE/antigen (Andrews et al., 2009). IgE-mediated allergic reactions cannot be established in FcεRIα or FcεRIβ-deficient mice (Ra et al., 2012; Taube et al., 2004). Without antigen, IgE alone can lead to low level receptor cross-linking, signal transduction, and mediator release (Kalesnikoff et al., 2001). Mast cell survival was also reported to be enhanced by IgE alone. The possible mechanism is through the activation of MAPK and Akt pathways. Mast cell responses mediated by tetrameric FcεRI have direct and in-direct effects on parasite killing by producing ROS and facilitate anti-parasitic functions of other immune cells, such as eosinophils, NK cells, and neutrophils.

1.2.3 FcεRI signaling in mast cells

The cross-linking of FcεRI by antigen through IgE can initiate a complex intracellular signaling cascade which results in mediator release. New adaptors, kinases, and negative regulators have been discovered continuously in recent years.

1.2.3.1 Initial phosphorylation

The first event after FcεRI cross-linking is the phosphorylation of ITAMs in the β and γ chains. It is generally accepted that the Src family protein tyrosine kinase Lyn, an acylated protein on membrane, is responsible for FcεRI phosphorylation (Bugajev et al., 2010).

Currently, three models for this process have been proposed (Bugajev et al., 2010). The first model is the transphosphorylation model. Lyn is non-covalently associated with a non-phosphorylated β chain, but ITAM in the β chain does not have access to the active site of Lyn. Receptor cross-linking allows Lyn to phosphorylate ITAMs of neighboring receptors. Another model, the lipid raft model, proposes that glycosylphosphatidylinisotol (GPI)-anchored proteins, and acylated proteins (such as Lyn) are concentrated in specific membrane regions termed as lipid rafts which are in a liquid-ordered state and provide a relatively stable platform for membrane signaling and trafficking (Sonnino and Prinetti, 2013). The lipids in a membrane may exist in three different states. In a solid-ordered state, lipid chains are tightly packed and move slowly. A liquid-disordered state is featured with loosely packed and fast-moving lipid chains. A liquid-ordered state is supposed to be more stable than the liquid-disordered state with more tightly organized and extended lipid chains (Heberle and Feigenson, 2011).The concept of lipid rafts will be described later in section 1.3. Lipid rafts enable the quick access and recruitment of elements needed for receptor signaling, such as receptors, membrane-bound kinases, and adaptors (Brown, 2006). In this model, FcεRI is considered to be separated from active Lyn residing in lipid raft domains of resting cells. After receptor cross-linking, aggregated FcεRI is close enough to be phosphorylated by

Lyn. The third model emphasizes the interplay of protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTP), which are known to be expressed by mast cells (Akimoto et al., 2009). PTP plays a counteractive role against PTK. The balance between PTK and PTP determines the phosphorylation status. In this model, PTP is considered as a guard maintaining the threshold of FcεRI tyrosine phosphorylation. After FcεRI cross-linking, PTP activity is suppressed, thus phosphorylation of ITAMs in β and γ chains can be catalyzed by PTK (Simons and Toomre, 2000). Superoxide and hydrogen peroxide, which are ROS and known inhibitors of PTP, have been shown to initiate early signaling events in the FcεRI pathway in mast cells (Heneberg and Draber, 2005).

1.2.3.2 Signal transduction

After initial phosphorylation of FcεRI, the signal cascade proceeds in the cytoplasm and involves interactions between kinases, adaptors, scaffolds, and small G proteins (Fig. 1.3). Phosphorylated ITAMs function as docking and activating sites for SH2 domains containing protein tyrosine kinases, mainly the Src family kinases Lyn and Fyn, as well as Syk family kinase Syk (Johnson et al., 1995). Fyn phosphorylates growth factor receptor-bound protein 2 (Grb2)-associated-binding protein 2 (GAB2), which facilitates activation of phosphoinositide 3-kinase (PI3K) (Xie et al., 2002). PI3K activation leads to phosphatidylinositol-3,4,5-trisphosphate (PIP3) production, which then recruits and activates protein kinase B (PKB, also known as Akt). Activated Akt then regulates activity of down-stream target proteins including B cell lymphoma-2 -associated death promoter (BAD), caspase-9, NF-κB, glycogen synthase kinase-3, and mammalian target of rapamycin (mTOR), which modulates cell proliferation,

differentiation, metabolism and apoptosis (Kim et al., 2008). PI3K also mediates activation of phosphoinositide-dependent protein kinase 1 (PDK1) and protein kinase C (PKC). PKC is responsible for degranulation and activation of NF-κB transcription factor (Windmiller and Backer, 2003). Syk activates PKC, as well as linker for activation of T cells (LAT) and the SH2 domain containing leukocyte protein of 76 kDa (SLP76) which are adaptor proteins forming large signaling complexes with other molecules such as the phospholipase C-γ (PLC-γ) (Siraganian et al., 2010). PLC-γ hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to produce second messengers diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). PLC-γ can also be activated by Bruton's tyrosine kinase (BTK), which is a downstream event of PIP3 production (Tkaczyk et al., 2003). DAG can also activate PKC (Teixeira et al., 2003). IP3 binds to receptors on the ER and causes calcium release from the ER which can activate PKC, contribute to degranulation, and activate nuclear factor of activated T-cells (NFAT), a transcription factor (Taylor and Thorn, 2001). LAT also mediates the activation of another adaptor protein, Grb2, and the guanine-nucleotide-exchange factor son-of-sevenless homologue (SOS) which leads to the activation of mitogen-activated protein (MAP) kinases (p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK)) and, ultimately, the activation of activator protein-1 (AP)-1 transcription factor (Saitoh et al., 2000). ERK can activate cytoplasmic phospholipase A2 (cPLA2) which frees arachidonic acid (AA, 20:4 n-6), eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) from phospholipids in plasma membranes (Fujishima et al., 1999). Eicosanoids can be

produced from AA or EPA under a series of oxidative enzymes, including cyclooxygenases (COX) and lipoxygenases (LOX) (Fig. 1.3) (Boyce, 2005).

FcεRI signaling can be regulated by inhibiting signals delivered by FcγRIIB, FcαRI and CD63. The IgG receptor FcγRIIB can recruit SH2-containing inositol 5-phosphatase (SHIP) through their immunoreceptor tyrosine-based inhibition motif (ITIM) (Ott et al., 2002; Tkaczyk et al., 2004). The IgA receptor FcαRI can inhibit FcεRI signaling by recruiting SHP-1, a SH2-containing tyrosine-specific protein phosphatase, which is expressed ubiquitously in the body (Pasquier et al., 2005). Antibodies against CD63, a tetraspanin molecule on the cell surface, were reported to inhibit mast cell degranulation by disrupting the GAB2-PI3K pathway (Kraft et al., 2005).

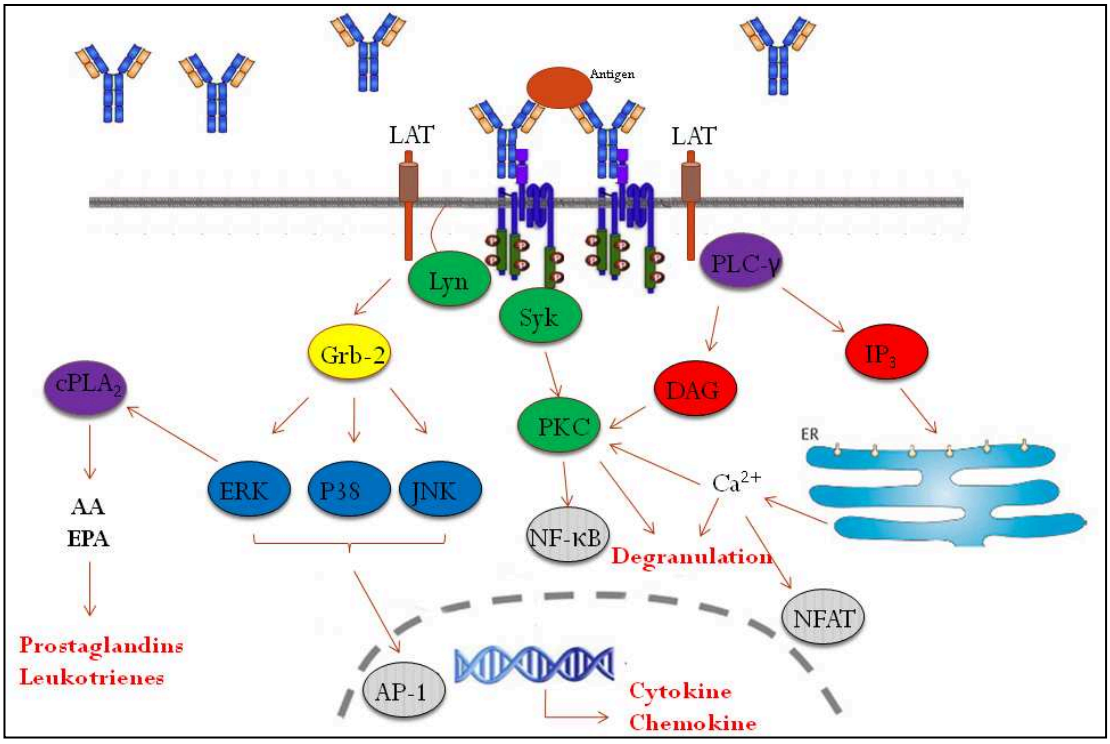

Figure 1.3 Diagram of FcεRI signaling. The binding of antigen to FcεRI-bound IgE causes receptor cross-linking, which leads to phosphorylation of ITAMs in β and γ chains by Lyn. Phosphorylated ITAMs function as docking and activating sites for Syk. Phosphorylated Syk then activates PKC, which is responsible for degranulation and activation of NF-κB. Syk also phosphorylates LAT, which facilitate the activation of PLC-γ to generate DAG and IP3 from PIP2. DAG activates PKC. IP3 causes calcium release from ER. LAT also activates another adaptor protein, Grb-2, which leads to activation of MAP kinases, ERK, JNK and P38. ERK activates $cPLA_2$ which free AA and EPA from plasma and nuclear membrane. Eicosanoids can be generated from AA and EPA. AA: Arachidonic acid; AP-1: Activator protein 1; $cPLA_2$: Cytosolic phospholipase A 2; DAG: Diacylglycerol; EPA: Eicosapentaenoic acid; ER: Endoplasmic reticulum; ERK: Extracellular signal-regulated kinase; Grb2: Growth factor receptor-bound protein 2; IP3: Inositol trisphosphate; ITAM: Immunoreceptor tyrosine-based activation motif; JNK: C-Jun N-terminal kinase; LAT: Linker of Activated T cells; NFAT: Nuclear factor of activated T-cells; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; PIP2: Phosphatidylinositol 4,5-bisphosphate; PKC: Protein kinase C; PLC: Phospholipase C; Syk: Spleen tyrosine kinase.

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1.3 Lipid rafts in mast cell biology

1.3.1 Concept of lipid rafts

Lipid rafts are specific membrane microdomains that contain high levels of cholesterol, sphingolipid, transmembrane proteins, and GPI-anchored proteins on the outer leaflet and acylated proteins on the inner leaflet (Fig. 1.4) (Calder and Yaqoob, 2007). Lipid rafts can be found in plasma membranes and intracellular membranes like the Golgi complex in all cell types (Nichols et al., 2001). Examples of GPI-anchored proteins that are associated with lipid rafts include CD14, CD80, and Thy-1, which are co-receptor, co-stimulatory molecule, and adhesion molecule (Clatza et al., 2003; Deininger et al., 2003; Schmitz and Orso, 2002). Lipid rafts function as platforms for membrane signaling and trafficking (Hanzal-Bayer and Hancock, 2007; Simons and Toomre, 2000). The plasma membrane is the place where cells detect signals from the extracellular environment. Lipid rafts are thought to facilitate the membrane signaling with harboured molecules such as receptors, adaptors, kinases and lipids required for signaling initiation and transduction. Membrane trafficking allows material transportation among cellular organelles, cell surface and extracellular environment (Cheung and de Vries, 2008). Fusion of lipid raft domains may contribute to this process (Alonso and Millan, 2001).

The classic fluid mosaic model of membranes has been challenged for years and some of the concept has been proven untrue. The distribution of membrane molecules seems to not be random, but is organized in some way. In the 1990s, low density detergent-resistant membrane regions were isolated (Brown and Rose, 1992), which formed the basis of the lipid raft concept. Lipid analysis has shown that lipid rafts are

rich in sphingolipid and cholesterol, and the phospholipids in lipid rafts contain higher levels of long chain saturated fatty acids compared to non-raft regions (Fig. 1.4) (Brown and London, 2000). As a result, lipid rafts are in a more ordered state than non-raft regions, and the fluidity is lower. Lipid rafts are characterized by a high melting temperature and insolubility in non-ionic detergents (London and Brown, 2000). Lipid rafts are also dynamic, which means their components are moving in and out of rafts constantly (Gupta and DeFranco, 2003). Lipid rafts are considered to be variable in terms of stability, size, shape, and composition (Rajendran and Simons, 2005).

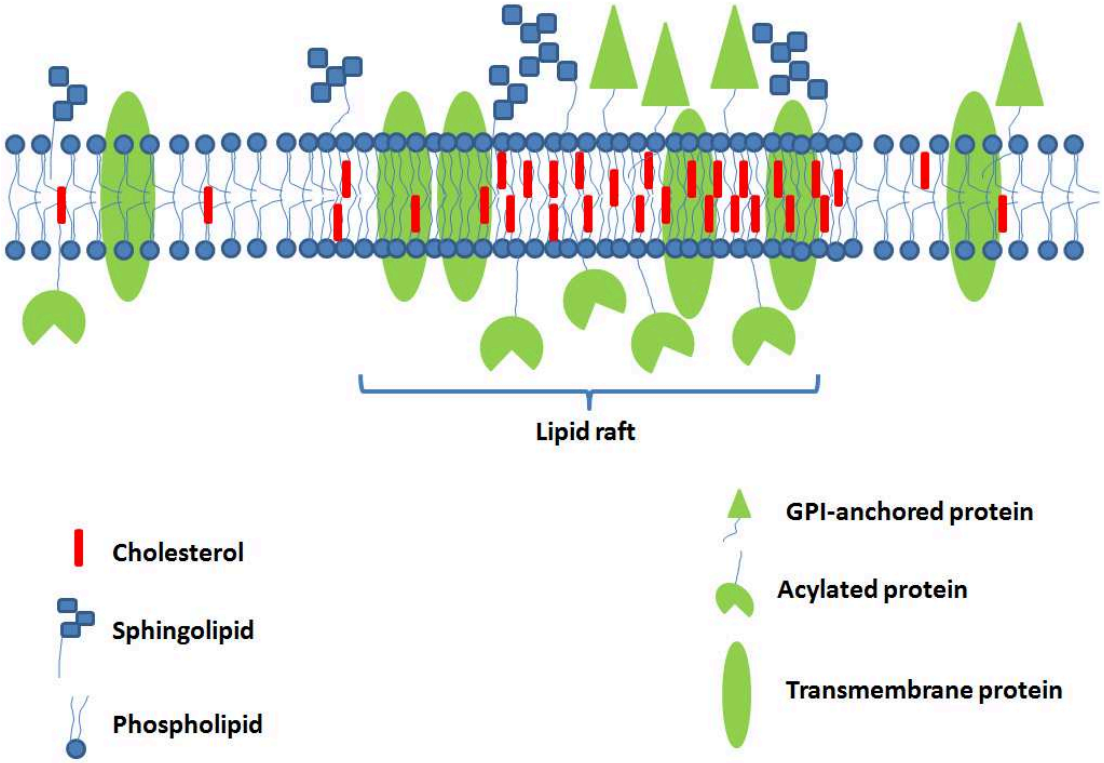

Figure 1.4 Structure of lipid rafts in cell membranes. Lipid rafts contain high levels of cholesterol, sphingolipid, transmembrane proteins, GPI-anchored proteins and acylated proteins. Rafts are considered to be packed more tightly than non-raft regions and are in a liquid-ordered state. GPI: glycosylphosphatidylinositol.

Some methods have been established to isolate and investigate lipid rafts. A common method to isolate lipid rafts is density gradient flotation using cold non-ionic detergents such as Triton X-100 based on their insolubility in non-ionic detergents (Brown, 2002). Flotillin, CD90 (Thy-1), Lyn, and monosialotetrahexosylganglioside (GM1) are common lipid raft markers used in research (Zajchowski and Robbins, 2002). The size of the rafts is estimated to be between 1 and 1,000 nanometres (Lingwood and Simons, 2010). Rafts can be visualized with fluorescence microscopy, typically using fluorophore-conjugated cholera toxin B to label GM1 (Brown, 2002). Western blot analysis is the most commonly used technique to detect protein expression in lipid rafts (Brown, 2002). Methyl-β-cyclodextrin (MBCD), a cholesterol-binding carbohydrate molecule, was used extensively to evaluate the role of lipid rafts in the biology of many cell types, including T cells, B cells, mast cells, breast cancer cells and more (Ostrom and Liu, 2007). However, MBCD is not specific for cholesterol in lipid rafts. Although rafts contain high level of cholesterol, cholesterol is actually distributed throughout the membrane and inside the cell. MBCD actually depletes all cholesterol inside the cell or on the cell membrane (Brown, 2002). Another cholesterol-depleting method is to use 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors like simvastatin to suppress cholesterol synthesis. Simvastatin is also not specific for cholesterol in lipid rafts (Nawarskas, 2005).

There is still debate on the existence and nature of lipid rafts because methods for isolating and studying rafts are limited which has restricted comprehensive research (Patel and Insel, 2009). One of the biggest arguments against lipid rafts is that the nature of the isolated material varies using different detergents or different concentrations of

the same detergent, leading to the possibility that artifacts are being created (Radeva and Sharom, 2004). However, detergent-free isolation of lipid rafts has been reported (Macdonald and Pike, 2005). Another controversy is that rafts were originally reported to be isolated using triton-X 100 in cold temperatures (4°C) only, but not at 37°C (London and Brown, 2000). However, the addition of cations was later found to allow the isolation of rafts at 37˚C (Chen et al., 2009). Using fluorescence microscopy, GPI-anchored folate receptors were shown to be localized in membrane regions that are not soluble in non-ionic detergents in live Chinese hamster ovary cells, indicating that rafts exist in living cells (Varma and Mayor, 1998). However, it is still not fully clear whether, or by how much, detergent expands pre-existing rafts.

1.3.2 Function of lipid rafts in immune cell signaling

One of the most important role of lipid rafts on the cell surface is mediating the signaling pathways initiated by antigens, growth factors, morphogens, and integrins (Simons and Toomre, 2000). Lipid rafts function as a platform for receptor signaling with concentrated molecules needed for signal transduction. In addition, inhibitory enzymes, such as phosphatases, which reside in non-raft regions, are blocked from influencing signaling (Simons and Toomre, 2000). Thus, lipid rafts allow for more efficient access of receptors to their ligands and facilitate further signal transduction. There are three models for the role of lipid rafts in the initiation of signaling (Simons and Toomre, 2000; Staubach and Hanisch, 2011). First, receptors and adaptors are concentrated in rafts. When the ligands are present, receptors will be cross-linked. Second, the presence of ligands results in the partitioning of receptors in lipid rafts, which facilitates the binding of ligands to their receptors. Third, clustering of receptors,

adaptors, or cytoskeleton elements causes the fusion of small rafts to form big rafts which have a greater accumulation of receptors, kinases, and adaptors.

The most extensively studied receptors in lipid raft research are T cell receptors (TCR), B cell receptors (BCR), and FcεRI, which are all multi-chain receptors with no intrinsic kinase activity in the intracellular tails, but which contain ITAMs. The mediating role of lipid rafts in the signaling of receptors with intrinsic kinase activity, such as the insulin receptor, was also shown in some studies (Morino-Koga et al., 2013). Besides signal transduction in adaptive immune cells, lipid rafts also function similarly in innate immune cells. TLR4 and CD 14, which is a GPI-anchored protein and functions as a co-receptor of TLR 4 to detect lipopolysaccharides (LPS), were found to be concentrated in lipid rafts of macrophages (Olsson and Sundler, 2006).

1.3.3 Role of lipid rafts in mast cells

Lipid rafts have been confirmed to be present in mast cells by numerous studies (Silveira et al., 2011). Some of the lipid raft components were reported to be important in maintaining mast cell morphology and mast cell development. Ganglioside (a raft component) deficiency leads to morphological changes of RBL-2H3 cells (Silveira e Souza et al., 2010) which are rat basophilic leukemia cells used extensively in mast cell research. After depletion of gangliosides, RBL-2H3 cells change from spindle to round shape and are smaller (Silveira e Souza et al., 2010). As membrane domains, there is compelling evidence showing that lipid rafts are involved in FcεRI-mediated endocytosis and signal transduction in mast cells, which will be discussed below.

1.3.3.1 Endocytosis

Receptors on cell membranes are constantly internalized and replaced by newly formed receptors (Gao et al., 2005). It was reported that ubiquitin ligases Cbl and Nedd4 are recruited into lipid rafts after mast cell activation mediated by FcεRI (Lafont and Simons, 2001). Cbl and Nedd4 may function to mediate FcεRI ubiquitination, which provides a sorting signal. Ubiquitin-tagged FcεRI is internalized through clathrin-coated vesicles and transported in the endosomes to the lysosomes for degradation (Fattakhova et al., 2009). Interestingly, in stimulated mast cells, GD1b (a ganglioside) in lipid rafts was shown to be internalized together with FcεRI in the same vesicle and follow the same path to the lysosomes (Oliver et al., 2007). In addition, GD1b deficiency suppressed receptor ubiquitination and endocytosis in mast cells (Mazucato et al., 2011). These results indicate that lipid rafts may not only facilitate the tagging of FcεRI for internalization, but also function as the site of vesicle formation.

1.3.3.2 Signal transduction

In stimulated cells, FcεRI was shown to be recruited to lipid rafts quickly and participate in the extensive protein-protein interactions in FcεRI signaling (Field et al., 1997). However, the other action models described above (section 1.3.2) may also exist. The models are not exclusive.

Many lipid raft components have been shown to regulate FcεRI signaling. Lyn is concentrated in lipid rafts and is more active in rafts than in non-raft regions, possibly because lipid rafts can protect Lyn from dephosphorylation by phosphatase (Young et al., 2003). Some raft components were found to regulate Lyn activity, such as flotillin-1. Flotillin-1 knockdown RBL-2H3 cells showed impaired Lyn activity with reduced

FcεRI phosphorylation, calcium mobilization, and ERK activation after cell activation through FcεRI (Kato et al., 2006). Besides protein components, lipid components in lipid rafts were also shown to modulate signaling. Cholesterol is required for phosphorylation of FcεRI. MBCD treatment significantly reduced tyrosine phosphorylation of FcεRI induced by IgE/antigen and suppressed mediator release from mast cells, possibly by disrupting the interaction between FcεRI and Lyn (Sheets et al., 1999). α-Galactosyl derivatives of ganglioside GD1b were found to down-regulate mast cell activation by promoting phosphatidylinositol hydrolysis and PKC redistribution (Field et al., 2000). These results indicate that lipid raft property has a great influence on FcεRI signaling and mast cell activation.

1.4 n-3 Polyunsaturated Fatty Acids (PUFAs)

1.4.1 Nomenclature, metabolism, and functions of fatty acids

Fatty acids are a group of hydrophobic molecules that are composed of hydrogen and carbon atoms with a methyl group at one end (ω or n end) of the molecule and a carboxyl group at the other end (Das, 2006a). If a fatty acid contains no carbon-carbon double bonds, it is called saturated. If a fatty acid contains one or more-than-one double bond, it is called a monounsaturated or PUFAs, respectively. Short-chain fatty acids contain fewer than six carbon atoms in the chain. Medium chain fatty acids contain 6–12 carbon atoms. The backbones of long chain fatty acids and very long chain fatty acids have 13-22 and more than 22 carbons atoms, respectively. The naturally formed double bond has cis configuration, which means hydrogen atoms on both side of the double bond are pointed in the same direction. Thus, double bonds in natural fatty acids cause

the molecule to kink in its molecular shape (Das, 2006b). According to the position of the first double bond from the n (ω) end, PUFAs can be classified as n-3, n-6 and n-9 PUFAs, whose first double bond is between the number 3 and 4, 6 and 7, 9 and 10 carbon from n (ω) end respectively (Fig. 1.5) (Simopoulos, 2000).

During digestion, free fatty acids are absorbed in the small intestine and transported to the blood through lymphatic vessels. Fatty acids are then taken up into cells by fatty acid-binding proteins and transported inside cells. Intracellular fatty acids can be transformed to acyl-CoA and carried by acyl-CoA binding protein to mitochondria or peroxisomes for ATP production. Acyl-CoA can also go to the endoplasmic reticulum for esterification to produce other types of lipids such as cholesterol, phospholipids, and triglycerides. Twenty carbon n-3 and n-6 PUFAs can be converted to eicosanoids by COX and LOX enzymatic pathways (Calder, 2010).

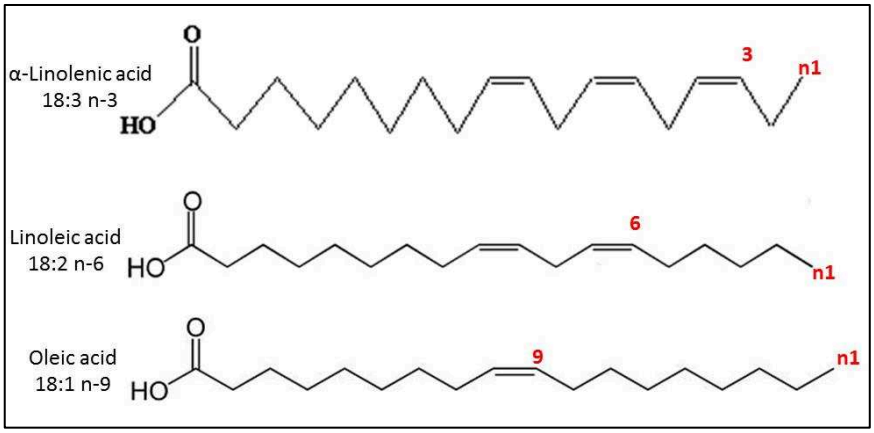

Figure 1.5 Representatives of n-3, n-6, and n-9 PUFAs. The position of first double bond from n (ω) end is between the number 3 and 4 carbon in n-3 PUFAs, 6 and 7 carbon in n-6 PUFAs, and 9 and 10 carbon in n-9 PUFAs.

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In general, fatty acids have four functions.

1) Three fatty acid chains and one glycerol form one molecule of triglyceride, which is the most efficient way to store energy in biology molecules. One gram of triglyceride stores 9 kcal energy. In contrast, one gram of protein and carbohydrate contain a similar amount of energy, 4 kcal. Alcohol can produce 7 kcal/g (Berg JM, 2002).

2) Fatty acids are building blocks for some biomolecules such as acylation of proteins and lipids forming the basic structure of membrane phospholipid bilayer (Holthuis and Levine, 2005). Thus, fatty acids are important for membrane structure and function.

3) Twenty carbon chain n-3 and n-6 PUFAs can be transformed under enzyme catalysis to eicosanoids, including PGs, LTs, thromboxanes, resolvins, protectins and lipoxins, which are signaling molecules modulating a broad range of body functions, such as body temperature, immune function, wound healing, pain, and smooth muscle relaxation/contraction (Calder, 2010; Fan et al., 2013).

4) Fatty acids can modulate gene expression directly. Free fatty acids can bind to certain G-protein-coupled receptors on the cell surface, initiate signaling, and modulate gene transcription. Fatty acids can also bind to peroxisome proliferator-activated receptor (PPAR) α and γ, which function together with retinoic-X receptors as nuclear receptors and regulate gene expression in inflammation, lipogenesis, lipoprotein assembly, β-oxidation, adipocyte differentiation, glucose metabolism, and insulin sensitivity (Varga et al., 2011).

1.4.2 n-3 PUFAs: effects and mechanisms

α-Linolenic acid (ALA) is the precursor of other n-3 PUFAs (Calder, 2013). ALA is an essential fatty acid, which means that it cannot be synthesized de novo in the body (Das, 2006a). EPA and docosahexaenoic acid (DHA) are the main bioactive forms of n-3 PUFAs in the body, generated from ALA by enzyme-catalyzed elongation, desaturation, and β-oxidation (Su, 2008). The structure of ALA, EPA and DHA was shown in Fig. 1.6. The n-3 PUFAs were listed in Table 1.2. In mammals, n-3 PUFAs cannot be synthesized de novo (Harel et al., 2001), and must be obtained from the diet or produced from ALA. Fish, shellfish, seed oil and nuts are good food resources of n-3 PUFAs (Deckelbaum and Torrejon, 2012).

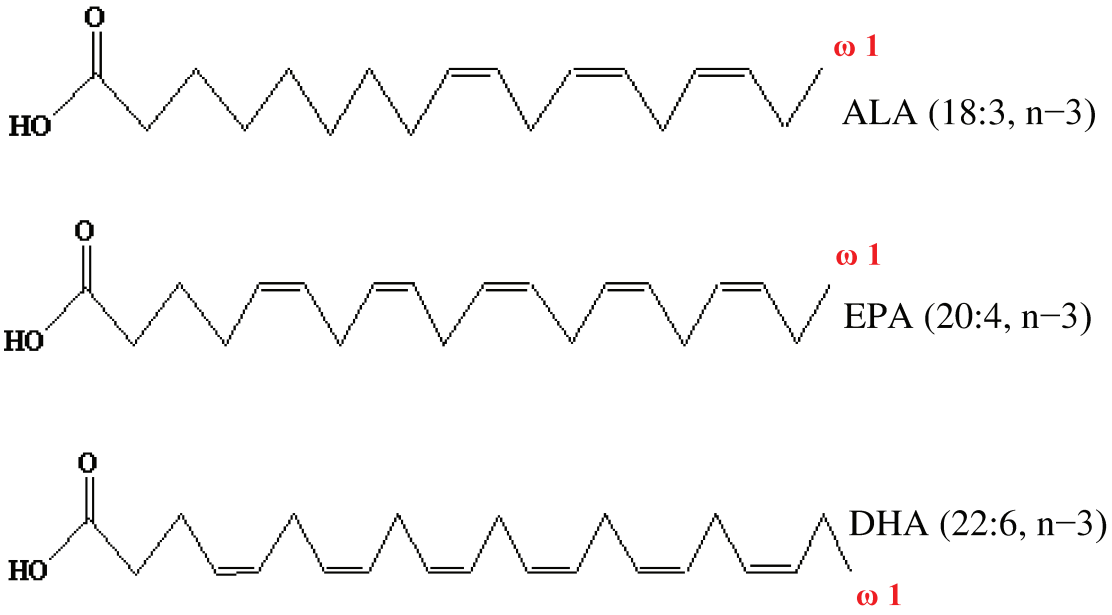

Figure 1.6 Structures of ALA, EPA and DHA. ALA, EPA and DHA contain 18, 20 and 22 carbon atoms respectively, with 3, 4 and 6 carbon-carbon double bonds. ALA: α-Linolenic acid. DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid.

Table 1.2 List of n-3 PUFAs

Common name	Lipid name	Chemical name	Formula/ molecular weight
Hexadecatrienoic acid	16:3 n-3	all-cis-7,10,13-hexadecatrienoic acid	$C_{16}H_{26}O_2$/250.376
α-Linolenic acid (ALA)	18:3 n-3	all-cis-9,12,15-octadecatrienoic acid	$C_{18}H_{30}O_2$/278.430
α-Parinaric acid	18:4 n-3	all-cis-9,11,13,15-octadecatetraenoic acid	$C_{18}H_{28}O_2$/276.414
Stearidonic acid	18:4 n-3	all-cis-6,9,12,15-octadecatetraenoic acid	$C_{18}H_{28}O_2$/276.414
Eicosatrienoic acid	20:3 n-3	all-cis-11,14,17-eicosatrienoic acid	$C_{20}H_{34}O_2$/306.483
Eicosatetraenoic acid	20:4 n-3	all-cis-8,11,14,17-eicosatetraenoic acid	$C_{20}H_{32}O_2$/304.467
Eicosapentaenoic acid (EPA)	20:5 n-3	all-cis-5,8,11,14,17-eicosapentaenoic acid	$C_{20}H_{30}O_2$/302.451
Docosapentaenoic acid (DPA)	22:5 n-3	all-cis-7,10,13,16,19-docosapentaenoic acid	$C_{22}H_{34}O_2$/330.504
Docosahexaenoic acid (DHA)	22:6 n-3	all-cis-4,7,10,13,16,19-docosahexaenoic acid	$C_{22}H_{32}O_2$/328.488

The health-promoting role of long chain n-3 PUFA, especially EPA and DHA, are well documented in a broad range of health and disease conditions. The consumption of EPA and DHA is associated with a lower risk of cancer, hyperlipidemia, cardiovascular diseases, hypertension, and neurodegenerative diseases (Siriwardhana et al., 2012). The low incidence of heart disease in the Inuit and Eskimos populations has been attributed to their high seafood intake, diets rich in n-3 PUFAs (Dewailly et al., 2001; Dyerberg et al., 1978; Holub, 1988). The regulatory functions of n-3 PUFAs on the immune system are also well known. N-3 PUFAs were reported to modulate activation of many immune cells, including T cells, B cells, mast cells, and basophils (Calder, 1997; Wu and Meydani, 1998). The multiple effects of n-3 PUFAs are the results of multiple mechanisms, summarized as follows:

1) As a membrane component, n-3 PUFAs are believed to regulate membrane structure and properties and, therefore cell function (Jump, 2002). EPA and DHA supplementation were reported to decrease AA and increase EPA/DHA levels in membranes, whole cells, and tissues, resulting in alteration in membrane structure and fluidity, protein distribution in membrane, and signal transduction (Anderson and Ma, 2009; Jump, 2002). In recent years, some studies have shown that n-3 PUFAs can reduce T cell response by altering lipid raft composition. In a study in human T cells, DHA supplementation was shown to increase ALA, docosapentaenoic acid (DPA, n-3), and DHA levels in lipid rafts, resulting in a decreased n-6/n-3 ratio in lipid rafts (Li et al., 2005). Subsequently, IL-2 receptor α (IL-2Rα) expression on cell surface was decreased. In addition, IL-2R α, β, and γ were relocated to non-raft regions after DHA treatment (Li et al., 2005). Furthermore, signal transducer and activator of transcription (STAT) 5a

and STAT5b recruitment to lipid rafts were inhibited (Li et al., 2005). In human breast cancer cells, EPA and DHA treatment decreased sphingomyelin, cholesterol, and DAG content and increased ceramide levels in lipid rafts (Schley et al., 2007). Epidermal growth factor receptor (EGFR) expression in lipid rafts was disrupted. EPA and DHA treatment inhibited breast cancer cell proliferation by over-promoting phosphorylation of both EGFR and p38 kinase, which function to activate AP-1 transcription factor (Schley et al., 2007).

2) EPA functions as a precursor for eicosanoids (Fig. 1.7). The main eicosanoid precursor in the body is AA. Eicosanoids from AA have high pro-inflammatory potential, while those from EPA have low pro-inflammatory potential (Calder, 2006). Eicosanoids can be made by oxidation of 20-carbon n-3 (EPA) and n-6 (AA and dihomo-γ-linolenic acid (DGLA)) PUFAs (Tapiero et al., 2002). However, 3-series PGs and 5-series LTs from EPA are weaker than AA-derived 2-series PGs and 4-series LTs at promoting vasodilation, smooth muscle contraction, vascular permeability, and leukocyte recruitment (Calder, 2006). E-series resolvins from EPA and protectins and D-series resolvins from DHA are anti-inflammatory (Fig. 1.7). In addition, there is competition between n-3 PUFAs and n-6 PUFAs as substrates for oxidation by COX and LOX, the critical enzymes for eicosanoid generation (de Roos et al., 2009), reducing the synthesis of highly pro-inflammatory eicosanoids from AA. PGs (1-series) from DGLA are anti-inflammatory, however the level of DGLA in body is about 10-fold lower than AA (Umeda-Sawada et al., 2006).

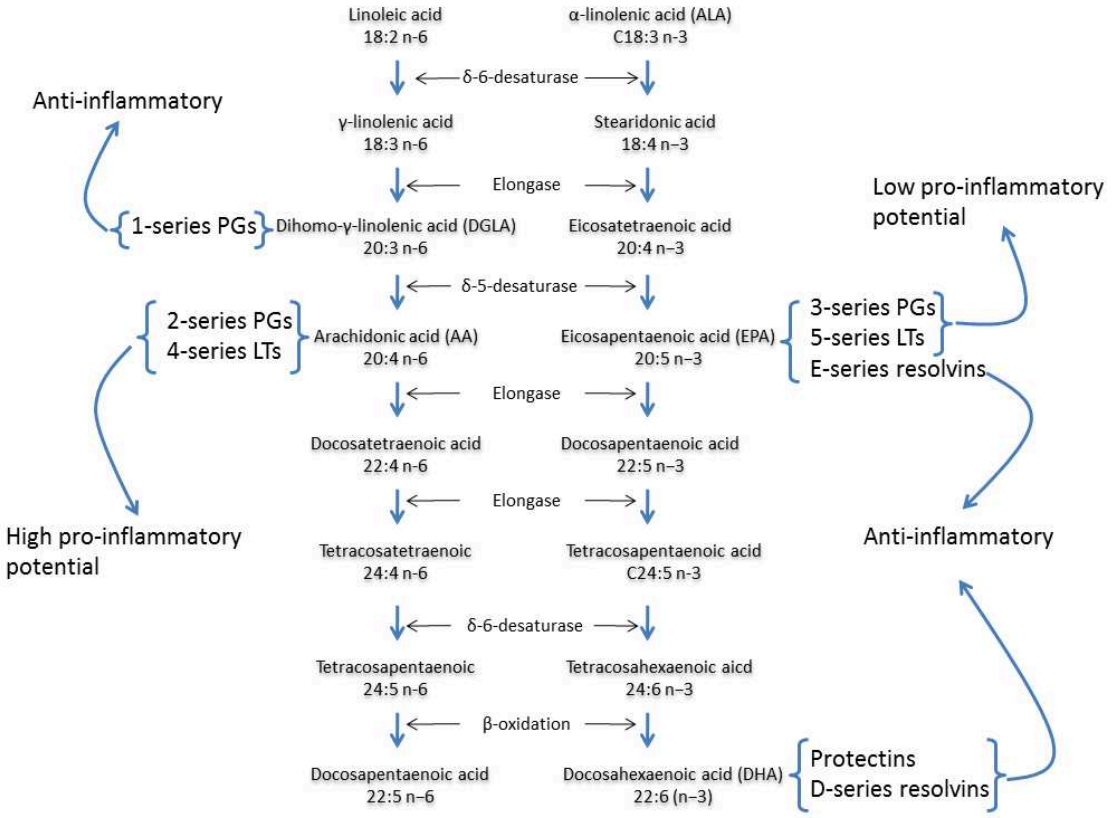

Figure 1.7 Eicosanoids derived from PUFAs. DGLA, AA and EPA are all precursors of eicosanoids. Two-series PGs and 4-series LTs from AA have high pro-inflammatory potential. Three-series PGs and 5-series LTs from EPA have low pro-inflammatory potential. One-series PGs from DGLA, E-series resolvins from EPA and protectins and D-series resolvins from DHA are anti-inflammatory; LTs: leukotrienes; PGs: prostaglandins.

3) Two cell surface receptors, both G-protein-coupled receptors, for n-3 PUFAs have been identified. G-protein-coupled receptor (GPR) 40 can bind to a broad range of medium and long chain free fatty acids (Briscoe et al., 2003; Mancini and Poitout, 2013). GPR40 is highly expressed in human brain and pancreas (Yonezawa et al., 2013). GPR40 has a regulating function in pain (Nakamoto et al., 2012) and insulin secretion of pancreatic β-cells (Salehi et al., 2005). In immune cells, GPR40 was only detected in monocytes (Mancini and Poitout, 2013).

GPR120 is another receptor to which long chain free fatty acids, with preference for PUFAs, bind (Oh and Olefsky, 2012). GPR120 is expressed in human adipose tissue, colon, lung, trachea, and macrophages (Oh and Olefsky, 2012; Oh et al., 2010). Using human epithelial colorectal adenocarcinoma cells Caco-2, GPR120 was shown to bind both n-3 (EPA, DHA) and n-6 (AA) PUFAs (Mobraten et al., 2013). EPA, DHA, and AA all enhanced intracellular calcium levels with similar efficacy, but the response of calcium to AA was quicker than DHA and slower than EPA. EPA, DHA and AA all activate ERK in Caco-2 cells, but AA had the slowest response (Mobraten et al., 2013). These results indicate that n-3 and n-6 PUFAs are all GPR120 ligands, but initiate similar signaling pathways with different potency. GPR120 was shown to modulate inflammatory responses. It was reported that GW9508, a GPR120 agonist, and DHA suppressed LPS-induced TNF and IL-6 production in RAW 264.7 cells (mouse leukaemic monocyte macrophage cells) and primary intraperitoneal macrophages (Oh et al., 2010). The effect was diminished after GPR120 knockdown.

4) n-3 PUFAs can activate or interfere with the activation of some transcription factors. They can also regulate gene transcription directly through binding to PPARs

(Zuniga et al., 2011) which are nuclear receptors that can be activated by a large range of fatty acids and fatty acid derivatives. PPARs regulate lipid metabolism, inflammation, immune function, and cell proliferation and differentiation (Peters et al., 2012). PPARs can also interact with NF-κB and limit its translocation to the nucleus. Thus, PPARs have an inhibiting effect on production of inflammatory mediators such as TNF and IL-1β (Chinetti et al., 2003). PPARs are expressed in adipose tissue, liver, pancreas, muscle, heart, macrophages, dendritic cells, lymphocytes, and mast cells (Linard and Souidi, 2010).

1.4.3 n-3 PUFAs, inflammation and allergy

In the last decade, many studies have been conducted to explore the relationship between n-3 PUFAs and inflammatory diseases. In a study conducted in rheumatoid arthritis patients, a significant improvement in symptoms was reported after 3 months of fish oil supplementation in terms of tender joint counts and duration of morning stiffness (Fortin et al., 1995). Besides reducing the production of pro-inflammatory eicosanoids from n-6 PUFAs, n-3 PUFAs were also found to be effective in inhibiting the synthesis of pro-inflammatory cytokines. DHA and ALA-enriched diet can reduce the mRNA expression of IL-1β, IL-6 in pancreas in a cerulein-induced pancreatitis model (Park et al., 2009). Fat-1 transgenic mice have a much lower n-6/n-3 fatty acid ratio in tissues because the mice are genetically modified to possess the ability to convert n-6 PUFAs to n-3 PUFAs (Kang, 2007). In dextran sodium sulfate-induced colitis model, the mRNA expression level of TNF in colons was found to be lower in fat-1 mice compared to wild type (WT) mice (Hudert et al., 2006).

A number of studies demonstrated the potential protective effects of n-3 PUFAs on allergic inflammation in human, especially allergic asthma. In a case-control study conducted in Australia, fatty acid intake data including n-6 and n-3 PUFAs were collected from 166 asthmatic and 169 normal children (Oddy et al., 2004). The difference between asthmatic and control subjects was determined after justifications for gender, age, breastfeeding, older siblings, maternal smoking during pregnancy, maternal age, maternal asthma, body mass index, total energy intake, and antioxidant intake. The logistic regression analysis revealed a positive association between the n-6/n-3 fatty acid ratio in diet and risk of asthma (Oddy et al., 2004). Some Northern European researchers did a cohort study on the relations between fish intake and asthma, which included 16,187 adult subjects aged 23-54 years (Laerum et al., 2007). Multiple logistic and cox regression analyses were conducted with adjustment for gender, adult hay fever, smoking, age, body mass index, household size, maternal smoking and family history of hay fever and asthma. Although no association between childhood fish intake and adult asthma was found, an elevated risk of asthma was shown in adults who did not consume fish as children (odds ratio=2.03) (Laerum et al., 2007). In a randomized placebo-controlled 3-year study (616 children at high risk of asthma enrolled), tuna fish oil supplementation significantly reduced the prevalence of cough in atopic children (Peat et al., 2004). This suggests that n-3 PUFAs may be effective in preventing the development of asthma in early childhood. In another double-blind human study, 5-weeks of n-3 PUFA-enriched fat blend (contains EPA, DHA, stearidonic acid (n-3) and γ-linoleic acid (n-6)) supplementation was reported to significantly decrease exhaled NO from young (22-29 years old) asthma patients challenged with low-dose dust mite

allergen, which caused the forced expiratory volume in 1 second to decreased by less than 5%. Blood eosinophil count and eosinophilic cationic protein were suppressed. The mite antigen-induced production of cysteinyl LTs (cys-LTs) by isolated leukocytes from these patients was also reduced (Schubert et al., 2009). These results indicate that the anti-allergic effect of n-3 PUFAs might be dependent on how n-3 PUFAs are provided in terms of dose, what specific kind of n-3 PUFAs were chosen, the age of subjects and the severity of disease. However, the association of n-3 PUFAs and reduced allergic inflammation seems to be clear, especially before the onset of diseases. Inhibited production of AA metabolites (cys-LTs) was suggested to be involved in protective effects of n-3 PUFAs on allergic inflammation (Schubert et al., 2009). Since mast cells are important effector cells in allergic inflammation that produce AA metabolites and mediate the early and late-phase reactions, n-3 PUFAs may down-regulate allergic inflammation by inhibiting mast cell activation.

1.4.4 n-3 PUFAs and mast cells

The suppressing effect of n-3 PUFAs on mast cell mediator release was indicated in some in vitro studies. EPA supplementation was reported to reduce PGD_2 production in mast cells derived from human umbilical cord mononuclear cells after IgE/anti-IgE challenge (Obata et al., 1999). Different types of n-3 PUFAs were also supplemented to animal mast cells to test their effect on cell responses. DHA was shown to inhibit PGE_2 production in C2 mast cells activated with the wasp venom peptide mastoparan (Gueck et al., 2004). ALA was reported to decrease tryptase activity, PGE_2 production, and histamine release from C2 cells activated in the same way (Gueck et al., 2004; Gueck et al., 2003). In the rat basophilic leukemia cell line RBL-2H3, ALA was shown to

decrease histamine release from cells activated with antigen and calcium ionophore A23187 (Kawasaki et al., 1994). However, the effect of n-3 PUFAs on mediator release from activated mast cells is still under debate because there are some issues on the analogy of the mast cell models and the different ways the cells were activated in studies introduced above. RBL-2H3 cells have a different membrane molecule profile from primary mast cells, and are considered to be closer to basophils (Passante et al., 2009). C2 cells may not express functional IgE receptors (Brazis et al., 2002). Mastoparan and A23187 activate mast cells by G protein activation and artificially increasing intracellular calcium levels, respectively. Both of these mechanisms are different to the FcεRI-mediated pathway, the principle pathway for mast cell activation (Siraganian, 2003). There are also studies with the opposite results showing that n-3 PUFAs potentiate mast cell mediator release, possibly due to model selection and the lack of controls over absorbed amount and the length of fatty acid treatment. For example, in RBL-2H3 cells, EPA dissolved in ethanol increased degranulation, TNF production, and Syk phosphorylation upon IgE/antigen stimulation after 48 hours (hr) treatment, in comparison to untreated cells (Nakano et al., 2005). However, RBL-2H3 cells are not a good model to study FcεRI signaling because the expression of FcεRI is not consistent in different labs using the same culture protocol (Froese et al., 1982).

The studies described above provided some indications that n-3 PUFAs may alter FcεRI-mediated mast cell activation because FcεRI signaling shares some down-stream signaling cascades with the pathways investigated by these studies, such as calcium mobilization (Fig. 1.3) induced by A23187, a calcium ionophore. However, the

previous studies presented little information on the mechanisms of how n-3 PUFAs regulate mast cell function. Thus, to evaluate the role and mechanisms of n-3 PUFAs in mast cell activation, a study using good mast cell analogues activated through FcεRI is needed.

1.4.5 n-3 PUFAs model systems

1.4.5.1 In vitro culture systems

N -3 PUFAs can be supplemented to cell culture as either PUFA sodium salts or pure PUFAs dissolved in organic solvent. N-3 PUFA sodium salt is water-soluble which is more convenient for dissolving in cell media. PUFAs can be liberated by hydrolysis. PUFA sodium salt supplementation can successfully result in PUFA incorporation into cells and cell membranes, which has been confirmed by previous study (Corsetto et al., 2011). PUFA sodium salts dissolved in water are generally stable for up to one year, considerably longer than pure PUFAs dissolved in dimethyl sulfoxide (DMSO) (6 months). Pure n-3 PUFAs are hydrophobic, requiring an organic solvent, such as DMSO, to be dissolved. The influence of DMSO on cell function also has to be considered, requiring additional controls.

The primary advantage of in vitro experiments is that the researchers can focus on the specific factor in which they are interested, while controlling other factors. In vitro studies are also generally less expensive and less time-consuming compared to in vivo studies. However, the results of in vitro studies are generated using isolated cells or a specific part of tissue/organ of the organism under highly controlled conditions. It is

always an issue to translate in vitro results to the biology of the intact organism and sometimes it is very easy to over-interpret the in vitro results.

1.4.5.2 In vivo animal model systems

n-3 PUFAs could be provided to animals either by food intake or intragastric administration. Dietary intervention is the natural way to take in n-3 PUFAs, but it is hard to control the amount of food an animal consumes. In addition, some confounding factors exist, including consistency of food preparation, food storage conditions, and feeding time and duration. It is much easier to control dose using intragastric administration. However, the damage to the esophagus is hard to avoid during studies, causing loss of appetite and potentially resulting in huge variation in some parameters at the end of the study.

Results from in vivo studies reflect the real circumstances in intact organisms. But, it is influenced by numerous factors which cannot be readily controlled. In addition, the end point of the study usually means sacrificing the animal, resulting in ethical considerations.

1.4.5.3 The fat-1 mouse model

Mammals normally cannot produce n-3 PUFAs de novo. The fat-1 transgenic mouse is genetically modified to express caenorhabditis elegans fat-1 gene encoding an n-3 fatty acid desaturase, which converts n-6 to n-3 PUFAs (Fig. 1.8) (Kang et al., 2004). The fatty acid profile data from fat-1 mice demonstrates significantly higher levels of n-3 PUFAs and lower levels of n-6 PUFAs than WT in many tissues including muscle, heart, brain, liver, kidney, lung, spleen, and even milk (Kang et al., 2004).

The fat-1 transgenic mouse has proven to be a model with unique merits (Kang et al., 2004). First, fat-1 mice have a reduced n-6/n-3 PUFA ratio with increased n-3 PUFA levels and decreased n-6 PUFA levels in tissue, which is greatly desirable for studying the effect of the n-6/n-3 PUFA ratio on health. Second, there is no need to prepare different diets for the different experimental groups, thus diminishing many confounding factors that could occur in a diet intervention study, as described above, and would lead to more reliable and definitive results. Third, fat-1 mice can be easily crossed with other disease models, including allergic asthma model (Bilal et al., 2011; Lu et al., 2008). The main disadvantage of fat-1 model is that gene knockin might result in unpredicted changes in phenotype.

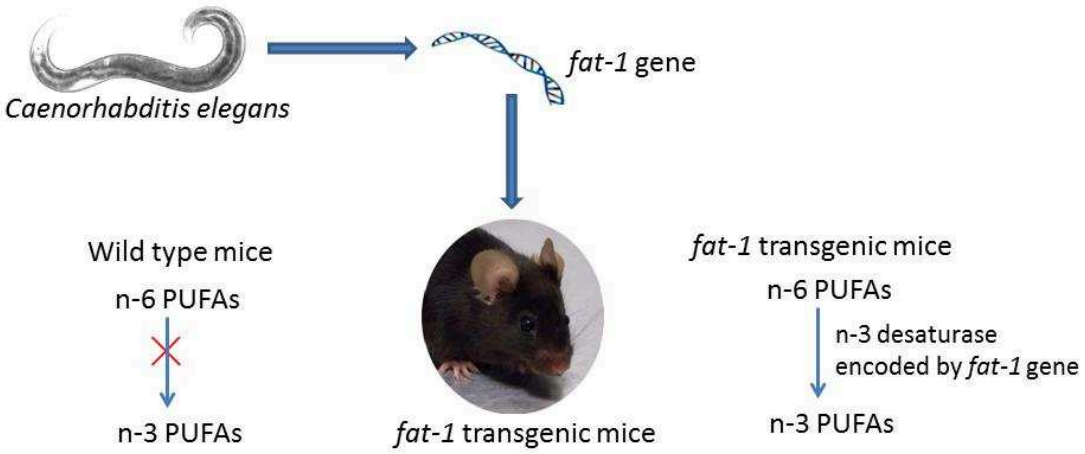

Figure 1.8 The fat-1 transgenic mouse model. Mammals cannot produce n-3 PUFAs de novo. While caenorhabditis elegans can convert n-6 PUFAs to n-3 PUFAs with an n-3 fatty acid desaturase encoded by fat-1 gene from Caenorhabditis elegans. Introducing of fat-1 gene to mice can enable the animal to produce n-3 PUFAs from n-6 PUFAs endogenously. PUFAs: Polyunsaturated fatty acids.

1.5 Rationale

n-3 PUFAs have beneficial effects against allergic inflammation. As a vital effector cell in allergic inflammation, mast cells are principally activated by IgE-binding antigen through FcεRI, which is believed to occur in lipid rafts formed by high levels of cholesterol, sphingolipids, phospholipids, and proteins. In a C2 canine mastocytoma cell line, n-3 PUFA supplementation increased n-3 PUFA levels in both raft and non-raft regions, but cholesterol levels did not change (Basiouni et al., 2012), indicating n-3 PUFA may regulate membrane structure and function. Thus, n-3 PUFAs may change the lipid profile of lipid rafts, affect lipid raft property, and disrupt signaling events dependent on lipid rafts, such as FcεRI-mediated signal transduction. The expression of some signaling molecules may also be altered by n-3 PUFA through binding to the free fatty acid receptors or PPARs (Calder, 2010).

1.6 General Hypothesis

n-3 PUFAs inhibit FcεRI-mediated signal transduction and mediator release in mast cells by inhibiting the expression of some molecules involved in FcεRI signaling and physically disrupting receptor association with lipid rafts.

1.7 Objectives

To determine the effect of both exogenous and endogenous n-3 PUFAs on FcεRI-mediated mast cell activation, two types of cells were used, mouse bone marrow-derived mast cells (BMMC) and laboratory of allergic diseases 2 (LAD2) human mast cells. BMMC are mucosal-type mast cells expressing functional FcεRI and kit on cell surfaces (Bischoff, 2007). They are IL-3 dependent and produce all

three groups of mediators under IgE/antigen challenge. BMMC are long lasting and the high yield could be easily achieved (Bischoff, 2007). LAD2 cells are connective-tissue mast cells that are dependent on SCF. They also express functional cell surface FcεRI (Bischoff, 2007). Increased n-3 PUFA levels were achieved in two ways. BMMC were cultivated from fat-1 transgenic mice which can produce n-3 PUFAs endogenously (section 1.4.5.3). Exogenously, n-3 PUFA sodium salts were supplemented to WT BMMC and LAD2 cells. The specific objectives of this project are to:

1) Characterize the effects of long chain n-3 PUFAs on FcεRI-mediated mast cell mediator release.

We hypothesized that n-3 PUFAs inhibit FcεRI-mediated mast cell activation. The fatty acid profile in mast cells will be measured by gas chromatography-mass spectrometry (GC-MS) to determine if the fatty acid profile is changed in fat-1 BMMC and long chain n-3 PUFA-supplemented BMMC. FcεRI-mediated mast cell degranulation, cys-LTs release, and cytokine/chemokine production will be measured by β-hexosaminidase (β-hex) release assay and enzyme-linked immunosorbent assay (ELISA) to determine if long chain n-3 PUFAs modulate mediator release of mast cells activated through FcεRI.

2) Examine the effects of long chain n-3 PUFAs on FcεRI expression and association with lipid rafts, FcεRI-mediated signal transduction, and expression of signaling proteins.

We hypothesized that long chain n-3 PUFAs suppress FcεRI-mediated signal transduction, disrupt FcεRI association with lipid rafts in resting and activated mast cells, and inhibit expression of some signaling proteins. Lipid rafts will be isolated by

sucrose gradient centrifugation for further protein and lipid analysis. The expressions of FcεRI on the cell surface, in whole cell lysates, and in lipid rafts will be measured by flow cytometry and western blot analysis to determine whether long chain n-3 PUFAs influence FcεRI expression on cell surface, in whole cell, and in lipid rafts. GC-MS will be used to determine if the fatty acid profile of the lipid rafts is altered in fat-1 BMMC. The expression and phosphorylation of Lyn, Syk, LAT after IgE/antigen stimulation will be measured by western blot analysis to see if long chain n-3 PUFAs modulate FcεRI-mediated signal transduction and expression of signaling proteins.

3) Evaluate the effects of lipid raft disruption on FcεRI-mediated activation of both WT and long chain n-3 PUFA-enriched mast cells.

We hypothesized that mast cell activation is diminished by lipid raft disruption in both WT and long chain n-3 PUFA-enriched mast cells. Lipid rafts of WT and fat-1 BMMC will be disrupted by depleting cholesterol with MBCD. Mast cell degranulation, cys-LT release and cytokine/chemokine production will be measured to determine the effect of lipid raft disruption on FcεRI-mediated activation of WT and fat-1 BMMC.

The measurement of mediator release represents mast cell activation. The results of flow cytometry and western blot analysis offer evidence to determine the effect of long chain n-3 PUFAs on FcεRI expression on cell surface, in whole cell lysates and rafts of resting cells, and the influence of long chain n-3 PUFAs on FcεRI shuttling in lipid rafts of activated cells and the expression and phosphorylation of signaling proteins. GC-MS analysis enables the evaluation of whether long chain n-3 PUFAs alter fatty acid composition of whole cells and rafts. This study could provide evidence on whether and how long chain n-3 PUFAs influence FcεRI-mediated mast cell

activation. This study could also clarify how exactly FcεRI mobilize in lipid rafts of mast cells, and the effect of long chain n-3 PUFAs on FcεRI localization and mobilization in lipid rafts of mast cells.

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CHAPTER 2

LONG CHAIN N-3 POLYUNSATURATED FATTY ACIDS MODULATE MAST CELL MEDIATOR RELEASE AND FATTY ACID PROFILE

2.1 Abstract

Aim of the study: The consumption of long chain n-3 polyunsaturated fatty acids (PUFAs) has been associated with reduced allergic inflammation. We hypothesized that long chain n-3 PUFAs inhibit mast cell activation induced through the high affinity immunoglobulin E (IgE) receptor, Fc ε receptor I (FcεRI).

Methodology: Bone marrow-derived mast cells (BMMC) and laboratory of allergic diseases 2 (LAD2) cultured human mast cells were supplemented with eicosapentaenoic acid (EPA, n-3), docosahexaenoic acid (DHA, n-3) and arachidonic acid (AA, n-6). Fat-1 transgenic BMMC were cultured from bone marrow of fat-1 transgenic mice. Gas chromatography-mass spectrometry (GC-MS) was used to evaluate lipid profiles. β-hexosaminidase (β-hex) release, cysteinyl leukotriene (cys-LTs) synthesis, and tumor necrosis factor (TNF) and CC chemokine ligand 2 (CCL2) production were measured by ELISA after IgE/2,4-dinitrophenyl- Human serum albumin (DNP-HSA) activation. Calcium mobilization was measured by fura-2 calcium imaging.

Results: Long chain n-3 PUFA levels were increased in fat-1 BMMC and BMMC supplemented with EPA and DHA compared to WT. EPA supplementation reduced β-hex release in LAD2 cells. Less β-hex, TNF, CCL2 and cys-LTs were produced by fat-1 BMMC. β-hex release,cys-LT synthesis and CCL2 production were also decreased in EPA-treated BMMC. DHA supplementation caused β-hex release and TNF, CCL2 production to decrease in BMMC. β-hex release from BMMC was also reduced in AA-treated cells. Calcium mobilization was decreased in fat-1 BMMC after cell activation.

Conclusion: Long chain n-3 PUFAs inhibit FcεRI-mediated mast cell mediator release.

2.2 Introduction

n-3 polyunsaturated fatty acids (PUFAs) are a subgroup of fatty acids containing more than one double bond with the first double bond located between the third and fourth carbons from the methyl end (Fig. 1.6). α-Linolenic acid (ALA) is the precursor of other n-3 PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the main active forms of n-3 PUFAs in the human body. EPA and DHA can be derived from ALA by enzyme-catalyzed elongation, desaturation, and β-oxidation (Fig. 1.7). Long chain n-3 PUFAs have a broad range of health benefits in humans. It is well established that diets rich in n-3 PUFAs are associated with lower risks of cancer, cardiovascular disease, neurodegenerative disease, hyperlipidemia, and hypertension (Abeywardena and Patten, 2011; Finocchiaro et al., 2012; Liu et al., 2011; Puri et al., 2005; Skulas-Ray et al., 2011).

Recently, the association between long chain n-3 PUFAs and allergic inflammation has been demonstrated in allergic asthma patients. For example, the dietary n-6/n-3 PUFA ratio is positively correlated with risk for asthma in children (Oddy et al., 2004), and long chain n-3 PUFA supplementation is protective against asthma in both asthmatic patients and in vivo animal asthma models (Schubert et al., 2009; Yokoyama et al., 2000). The ability of long chain n-3 PUFAs to inhibit some of the symptoms associated with asthma has been attributed to decreased 4-series leukotriene (LT) and increased 5-series LT production by activated leukocytes (Schubert et al., 2009), decreased eosinophil and neutrophil infiltration in bronchoalveolar lavage fluid (BALF) (Yokoyama et al., 2000), decreased production of pro-inflammatory cytokines such as interleukin (IL)-1α, IL-5, IL-9, IL-13, tumor necrosis factor (TNF), granulocyte/

macrophage colony-stimulating factor (GM-CSF), CC chemokine ligand 5 (CCL5) in BALF, and increased expression of protectin D1 and resolvin E1 in the lungs (Bilal et al., 2011). Mast cells are known to be key effector cells that mediate allergic inflammatory responses (Theoharides and Kalogeromitros, 2006). The connection between long chain n-3 PUFAs and reduced mast cell activation was suggested by some studies. For example, EPA was reported to inhibit prostaglandin (PG) D_2 production by mast cells activated by immunoglobulin E (IgE)/anti-IgE (Obata et al., 1999). However, the effect of long chain n-3 PUFAs on mast cell activation is still poorly understood.

Mast cells can be activated by allergens, complements, antimicrobial peptides, adenosine, neuropeptides and physical stimuli (Galli et al., 2005). The principle allergen-induced activation pathway is mediated by the high affinity IgE receptors, Fc ε receptor I (FcεRI) (Gilfillan and Tkaczyk, 2006). FcεRI typically has a heterotetrameric structure (αβγ2) composed of one α subunit with an extracellular domain for IgE binding, a four-transmembrane-spanning β subunit, and two identical disulphide linked γ subunits (Kraft and Kinet, 2007) as shown in Fig. 1.1. The cross-linking of IgE-bound FcεRI by antigen leads to mast cell degranulation resulting in release of preformed mediators such as histamine, synthesis of lipid-derived mediators such as LTs and PGs, and cytokine/chemokine production (Hofmann and Abraham, 2009).

In this study, I hypothesized that long chain n-3 PUFAs can inhibit mast cell mediator release induced by IgE/antigen. Long chain n-3 PUFAs were supplemented to mouse and human mast cells. I also cultivated bone marrow-derived mast cells (BMMC) from fat-1 transgenic mice which have elevated long chain n-3 PUFA levels compared to wild type (WT) mice. The fat-1 gene encodes a fatty acid n-3 desaturase from C. elegans

and allows fat-1 transgenic mouse to convert n-6 to n-3 PUFAs, resulting in endogenous conversion of n-6 PUFAs to n-3 PUFAs (Kang et al., 2004) (Fig. 1.8). In wild type (WT) mice (as with all mammals), n-3 PUFAs cannot be synthesized de novo; they must be consumed through diet. The effects of long chain n-3 PUFAs on mast cell activation were evaluated in the context of degranulation, cysteinyl leukotriene (cys-LT) release, and cytokine/chemokine production.

2.3 Materials and Methods

2.3.1 Animals and diet

The femurs of WT and fat-1 mice were provided by Dr. David W.L. Ma, University of Guelph (Guelph, ON). With the approval from the University of Guelph Animal Care Committee, fat-1 mice were obtained by breeding C57BL/6 × C3H fat-1 males (provided by Dr. Jing X. Kang, Harvard Medical School, Boston, MA) with wild type (WT) C57BL/6 females (Charles River, Saint-Constant, QC). WT and fat-1 mice were all housed in standard clean environmental conditions with controlled temperature and humidity. An AIN-93G diet supplemented with 10% linoleic acid-enriched safflower oil (Research Diets, New Brunswick, NJ) was provided ad libitum to all animals.

2.3.2 Growth of human and mouse mast cells

Laboratory of allergic diseases 2 (LAD2) human mast cells were cultured in StemPro-34 SFM media (Life Technologies, Burlington, ON) supplemented with 2 mm L-glutamine (Corning cellgro, Manassas, VA), 100 U/mL penicillin/100 μg/mL streptomycin (Corning cellgro) and 100 ng/mL stem cell factor (Life Technologies). Cells were fed every 4-5 days.

BMMC were cultivated from the bone marrow of WT and fat-1 transgenic mice. Cells were maintained in RPMI-1640 media (Corning cellgro) supplemented with 4 mM L-glutamine (Corning cellgro), 50 μM BME (Sigma-Aldrich, Oakville, ON), 1 mM Na pyruvate (Corning cellgro), 100 U/mL penicillin/100 μg/mL streptomycin (Corning cellgro), 0.1 mM nonessential amino acids (Corning cellgro), 25 mM HEPES (Corning cellgro), 10% FBS (Gibco, Burlington, ON) and 10 ng/mL mouse recombinant

interleukin (IL)-3 (BioLegend, San Diego, CA). Cells were fed every 3-5 days. After 4 to 5 weeks, cell purity was determined by measuring expression of cluster of differentiation (CD)117 (c-Kit) and FcεRI by flow cytometry. After 5 weeks, 99% of the cells were double positive for CD117 and FcεRI. The cell viability was monitored using trypan blue staining (Sigma-Aldrich).

2.3.3 Toluidine blue staining

Slides were prepared using Shandon Cytospin Cytocentrifuge (Thermo Fisher Scientific Inc, Waltham, MA) with 1×10^5 cells. Cells on the slide were then fixed with Mota's fixative containing 4% lead acetate basic (Sigma-Aldrich) for 15 minutes (min). After washing and drying, cells were stained with 0.5% toluidine blue (Sigma-Aldrich) for 20 min.

2.3.4 Flow cytometry

Briefly, cells were washed with phosphate-buffered saline (PBS) containing 0.1% BSA (Sigma-Aldrich) and re-suspended in PBS containing 1.55% BSA (2×10^6 cells/mL), and then incubated in 2 μg/mL rat anti-mouse CD117 (c-Kit) phycoerythrin (PE)-Cy7 (eBioscience, San Diego, CA) and 3 μg/mL armenian hamster anti-mouse FcεRIα PE (eBioscience) for 1 hour (hr). After washing twice with 0.1% BSA in PBS, cells were re-suspended in 200 μl 0.1% BSA in PBS and transferred to a round bottom 96-well plate. Cell samples were analyzed on a FACS Array flow cytometer (BD Biosciences, Mississauga, ON). Rat IgG2bK PE-Cy7 (eBioscience) and armenian hamster IgG PE (eBioscience) were used as isotype controls. Data was generated using WinMDI 2.9 software.

2.3.5 Fatty acid supplementation to BMMC and LAD2 cells

LAD2 cells were supplemented with 0.1, 1, 10, 100 and 500 μM EPA-Na, DHA-Na, or arachidonic acid (AA)-Na for 24 hr and 48 hr at 5× 10^5 cells/mL. WT BMMC were spun down and resuspended in fresh media at 5× 10^5 cells/mL. EPA-Na (Sigma-Aldrich), DHA-Na (Sigma-Aldrich), or AA-Na (Sigma-Aldrich) in PBS was supplemented to cell cultures for 24 hr in a final concentration of 100 μM, as indicated by the results gained in LAD2 cells.

2.3.6 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay

After fatty acid supplementation to BMMC and LAD2 cells, cell viability was measured using a XTT proliferation kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the instructions from the manufacturer.

2.3.7 RNA isolation, cDNA synthesis, and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from BMMC using TRI reagent (Sigma-Aldrich). First strand cDNA was generated from 1 μg RNA with the mixture of 200 units Moloney murine leukemia virus reverse transcriptase (Life Technologies), 500 μg/mL oligo (dT) primer (Promega, Madison, WI), 10 mM dNTP Mix (Promega), 0.1 M DTT (Life Technologies), and 5× first strand buffer (Life Technologies).

RT-PCR was performed with 2 μl of cDNA reaction mixed with 10x PCR Buffer (Life Technologies), 50 mM $MgCl_2$ (Life Technologies), 10 mM dNTP mix (Life Technologies), 5 U/μL Taq DNA polymerase (Life Technologies), forward primer

5′-TGTTCATGCCTTCTTCTTTTTCC-3′ and reverse primer 5′-GCGACCATACCTCAAACTTGGA-3′ for the fat-1 transgene; forward primer 5′-GTGTTCCTACCCCCAATGTG-3′ and reverse primer 5′-GGTCCTCAGTGTAGCCCAAG-3′ for glyceraldehydes 3-phosphate dehydrogenase (GAPDH) using MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) at the following cycling program: 94°C for 2 min (1 cycle); 94°C for 30 s, 51°C for 30 s, 72°C for 30 s (35 cycles); and 72°C for 7 min (1 cycle). The PCR products were analyzed with 1.5% agarose electrophoresis, followed by exposing gel to ultraviolet (UV) light in a ChemiDoc XRS system (BD Biosciences) for image acquisition.

2.3.8 Gas chromatography–mass spectrometry (GC-MS)

Lipid was extracted and methylated by the method modified from a previous study (Kang and Wang, 2005). Briefly, 6×10^6 BMMC were mixed with 1 mL hexane and 1 mL 14% BF_3/MeOH. After blanketing with nitrogen, samples were heated at 100°C for 1 hr. One milliliter H_2O was added prior to centrifugation at 200× g for 1 min. The upper hexane layer containing fatty acid methyl esters (FAMEs) was collected for GC-MS analysis.

GC-MS was performed in an Agilent 6890N network GC system (Agilent Technologies, Mississauga, ON) with a split/splitness injector (Agilent Technologies) (Agilent Technologies). Fatty acid components were detected by a flame ionization detector (Agilent Technologies) and a MS detector (Agilent Technologies). A supelco 37 component FAME mix standard (Sigma-Aldrich) was used to check the performance

of the GC instrument. Samples were analyzed using DB-23 column (Agilent Technologies) with helium as the carrier gas at a flow rate of 1.0 ml/min.

2.3.9 Degranulation assay

BMMC were first incubated with 500 ng/mL mouse anti-2,4-dinitrophenyl (DNP) IgE (Sigma-Aldrich) at 5×10^5 cells/mL in complete media for 24 hr for sensitization. Cells were then re-suspended in HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.4 mM $Na_2HPO_4{\cdot}7H_2O$, 5.6 mM glucose, 1.8 mM $CaCl_2{\cdot}2H_2O$, 1.3 mM $MgSO_4{\cdot}7H_2O$) and transferred to a round bottom 96-well plate. Finally, cells were treated with DNP-human serum albumin (DNP-HSA, Sigma-Aldrich) in varying concentrations of 0.001, 0.01, 0.1, 1, 10, 100, 200, 500 ng/mL for 30 min for stimulation. LAD2 cells were sensitized with 500 ng/mL IgE-biotin (BioLegend) for 24 hr, followed by stimulation with 500 ng/mL streptavidin (Sigma-Aldrich) for 30 min.

After sensitization and stimulation, mast cell degranulation was assessed by measuring β-hexosaminidase (β-hex) release. Briefly, each cell suspension was spun at 200× g for 5 min, and the supernatant and pellet fraction were separated. Fifty microliter of supernatant was incubated with 1 mM β-hex substrate 4-nitrophenyl N-acetyl-β-D-glucosaminide (Sigma-Aldrich) in 0.04 M citrate buffer (pH 4.5) at 37°C for 1.5 hr. Fifty microliter of the pellet fraction was suspended with 0.1% Triton X-100 for 2 min at room temperature, and then incubated with 1 mM 4-nitrophenyl N-acetyl-β-D-glucosaminide at 37°C for 1.5 hr. After adding 50 µl 0.4 M glycine (pH10.7) to both supernatant and pellet plates, absorbance was measured at A_{405} and A_{570} in a Multiskan Ascent 354 microplate reader (Thermo LabSystems, Vantaa,

Finland). Data was generated by using the following formula: % release = 100 × supernatant absorbance/(0.5 × supernatant absorbance + pellet absorbance).

2.3.10 Enzyme-linked immunosorbent assay (ELISA) for cys-LTs, TNF, and CC chemokine ligand 2 (CCL2)

BMMC were first sensitized for 24 hr with 500 ng/mL mouse anti-DNP IgE (Sigma-Aldrich). The cell suspension was transferred to a 6-well plate. After stimulating cells with DNP-HSA (Sigma-Aldrich) in varying concentrations of 1, 10, 100, 200, 500 ng/mL for 6 hr at 37°C for cytokine/chemokine production or 3 hr at 37°C for cys-LT release, supernatant was collected for ELISA measurements by centrifuging the cell suspension at 200× g for 5 min.

The supernatant was analyzed for TNF, CCL2, and cys-LT release using commercial ELISA kits (eBioscience; Enzo Life Sciences, Farmingdale, NY) according to the manufacturers' protocols.

2.3.11 Calcium mobilization

WT and fat-1 BMMC were first sensitized with 500 ng/mL anti-DNP IgE for 24 hr. Cells were then loaded with 1 μM fura-2 AM (Invitrogen) in HEPES buffer for 30 min in the dark. After washing, cells were seeded in a concentration of 3×10^6 cells/mL in a glass-bottomed culture dish coated with 10 mg/mL poly-D-lysine (Sigma-Aldrich) overnight. The calcium response was recorded using an inverted microscope (IX71, Olympus Canada Inc., Toronto, ON). Fura-2 was excited at 340 and 380 nm. Excitation of calcium-bound fura-2 at 340 nm leads to increased fluorescence emission. In contrast, excitation of fura-2 at 380 nm leads to decreased fluorescence emission. So the ratio of

fluorescence emission 340/380 indicates calcium concentration, and the influence of difference in fura-2 concentration and cell thickness are ruled out. DNP-HSA was added at the time point of 20 s in concentration of 10 ng/mL. The response of 20 randomly selected cells was analyzed using SlideBook 4.0 software (Olympus).

2.3.12 Statistical analysis

The differences of fatty acid profile between WT and fat-1 BMMC, and between groups of PUFA-treated BMMC were determined using Student's t-test and one-way analysis of variance (ANOVA) followed by the post-hoc Bonferroni test. Two-way ANOVA followed by the post-hoc Bonferroni test was used to evaluate the differences in mediator release between WT and fat-1 BMMC, and among PUFA-treated BMMC. Statistical significance was set at $p<0.05$. Statistical analysis was performed using SPSS 11.5 statistical software package (IBM Corporation, Armonk, NY).

2.4 Results

2.4.1 BMMC and LAD2 genotyping and phenotyping

Toluidine blue stained BMMC and LAD2 cells were round and nuclei were big with clear boundaries. Granules were clearer in the cytoplasm of BMMC and LAD2 cells (Figs. 2.1 and 2.2). After 4 weeks of culture, more than 99% of BMMC were double positive for the mast cell markers CD117 and FcεRI (Fig. 2.3).

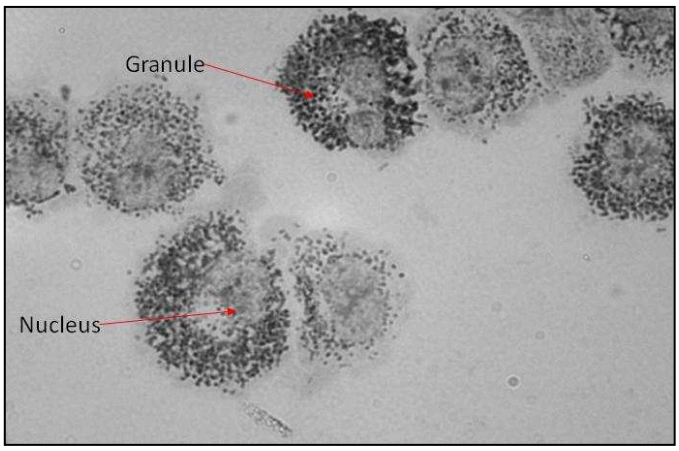

Figure 2.1 Toluidine blue staining of BMMC (100 × magnification)

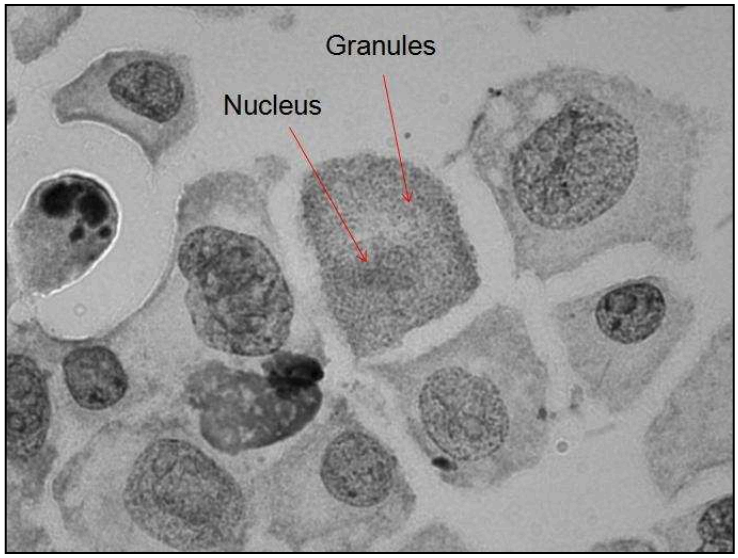

Figure 2.2 Toluidine blue staining of LAD2 cells (100 × magnifications)

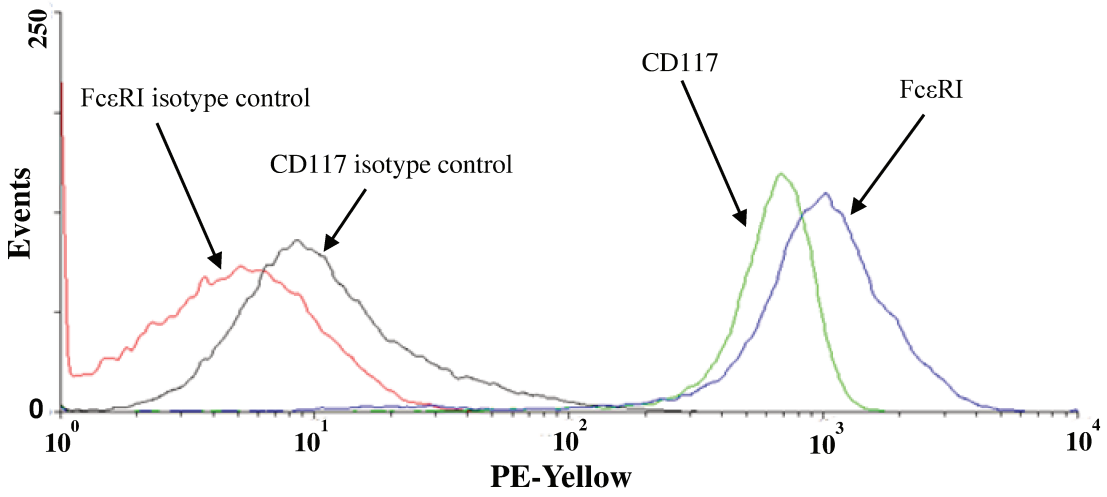

Figure 2.3 The expression of CD117 and FcεRI on cell surface of BMMC after 4 weeks culture. The surface expression of CD117 and FcεRI was detected by flow cytometry together with appropriate isotype controls. PE: phycoerythrin. Events on the y-axis represent cell counts.

2.4.2 The effect of long chain n-3 PUFA supplementation on mast cell viability

The XTT assay showed that 24 hr and 48 hr treatments of 0.1, 1, 10, 100 μM EPA-Na, DHA-Na, AA-Na did not influence LAD2 cell viability. However, 500 μM EPA-Na, DHA-Na, AA-Na supplementation for 24 hr and 48 hr all significantly reduced LAD2 cell viability by >50% (all, $p<0.001$, Fig. 2.4). After 24 hr, 100 μM of EPA-Na, DHA-Na, and AA-Na supplementation was not cytotoxic to BMMC (Fig. 2.5).

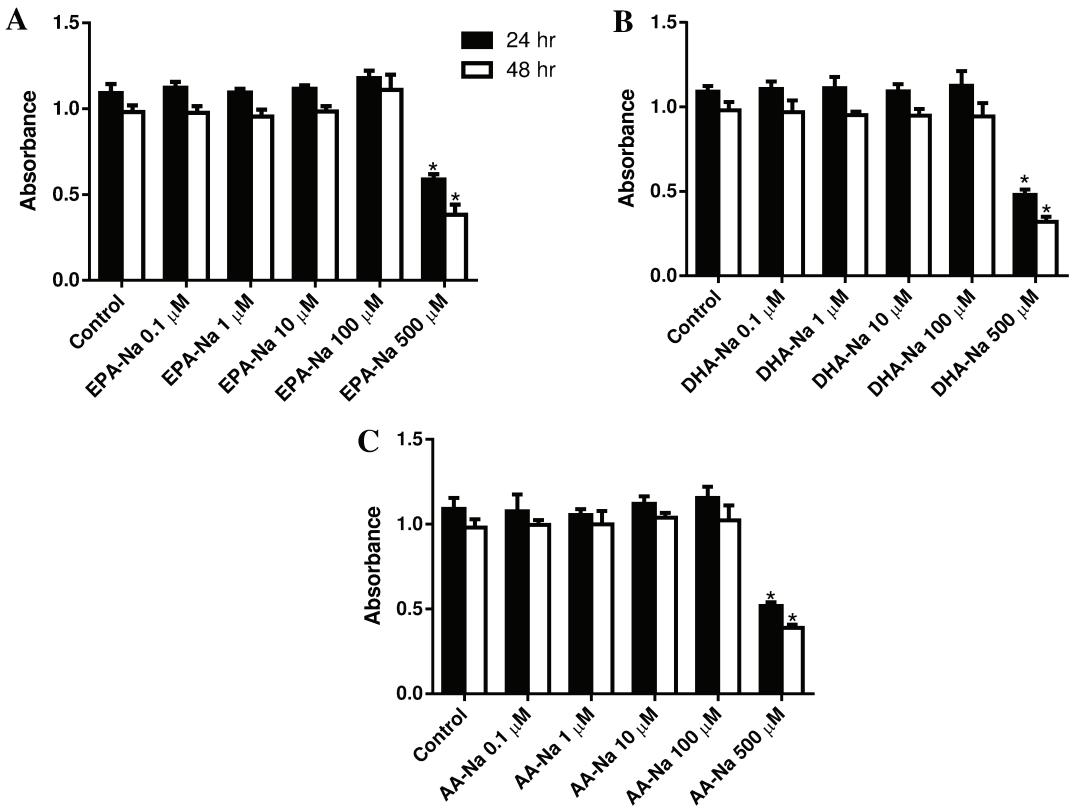

Figure 2.4 The effect of long chain PUFA supplementation on cell viability of LAD2 cells. LAD2 cells were treated with indicated concentrations of EPA-Na (A), DHA-Na (B), and AA-Na (C) for 24 hr and 48 hr. Cell viability was measured by the XTT assay. *: $p<0.001$, compared to untreated controls, one-way ANOVA followed by the post-hoc Bonferroni test. Error bars represent standard error of the mean (SEM) (n=3). XTT: 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

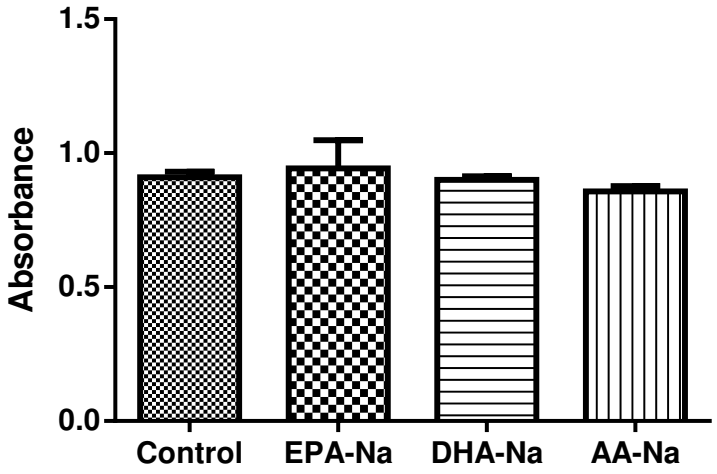

Figure 2.5 The effect of long chain PUFA supplementation on cell viability of BMMC. BMMC were treated with 100 μM EPA-Na, DHA-Na, and AA-Na for 24 hr. Cell viability was measured by XTT assay and is compared to untreated controls. Error bars represent SEM (n=3). XTT: 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5- carboxanilide.

2.4.3 The effect of long chain n-3 PUFA supplementation on n-3 PUFA levels of BMMC

EPA-Na treatment increased EPA level ($p=0.004$) and docosapentaenoic acid (DPA) level ($p=0.007$) of BMMC. DHA-Na treatment increased DHA level ($p=0.044$). AA-Na treatment increased AA level ($p=0.001$) (Figs. 2.6-2.9, Table 2.1). Compared to untreated cells, significant lower n-6/n-3 ratios were seen in EPA-Na ($p=0.034$) and DHA-Na ($p=0.027$) treatments. AA-Na supplementation resulted in a much higher n-6/n-3 ratio ($p<0.001$) than untreated cells.

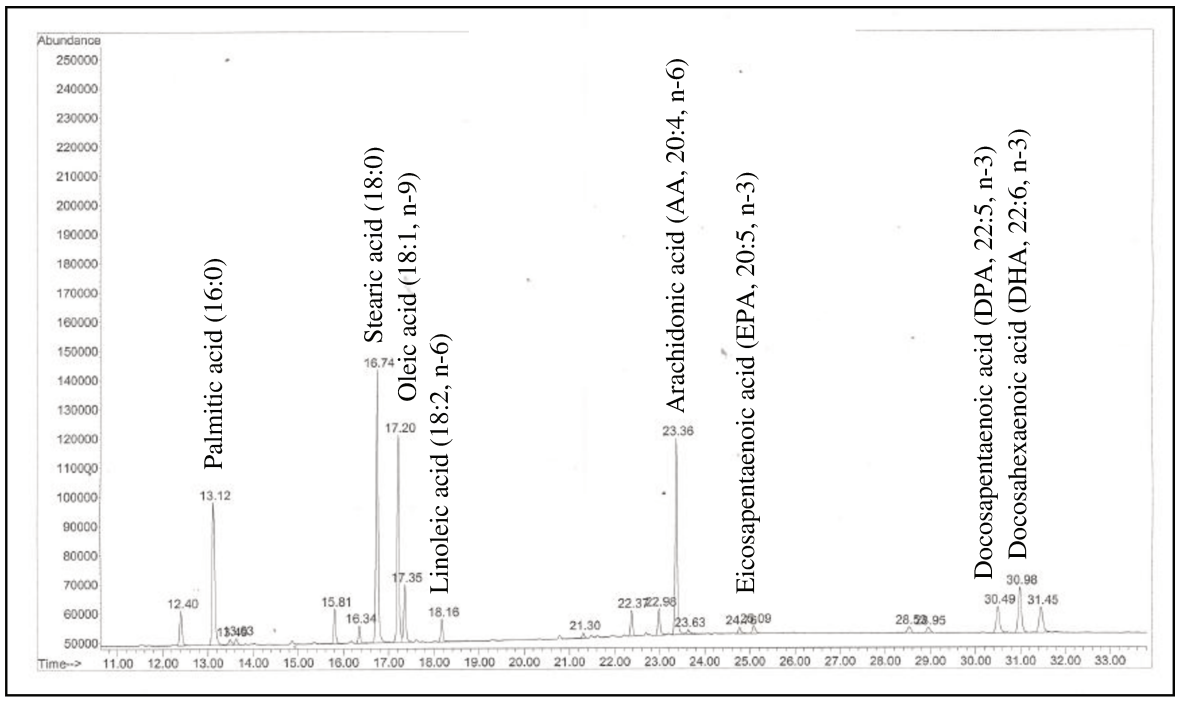

Figure 2.6 Fatty acid profile in untreated BMMC. Eight fatty acids were identified by GC-MS analysis, which were marked besides the peaks. Data is the representative of three independent experiments with similar results.

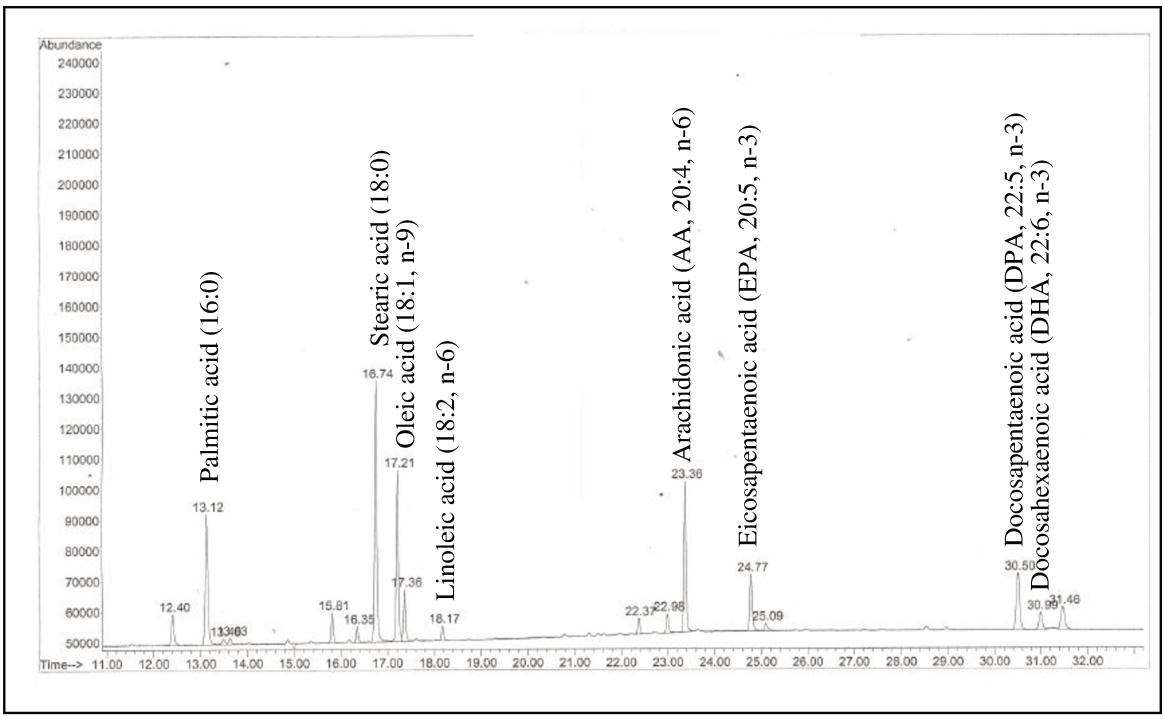

Figure 2.7 Fatty acid profile in 100 µM EPA-Na-treated BMMC. After 24 hr treatment with 100 µM EPA-Na, the fatty acid profile was determined by GC-MS. Data is the representative of three independent experiments with similar results.

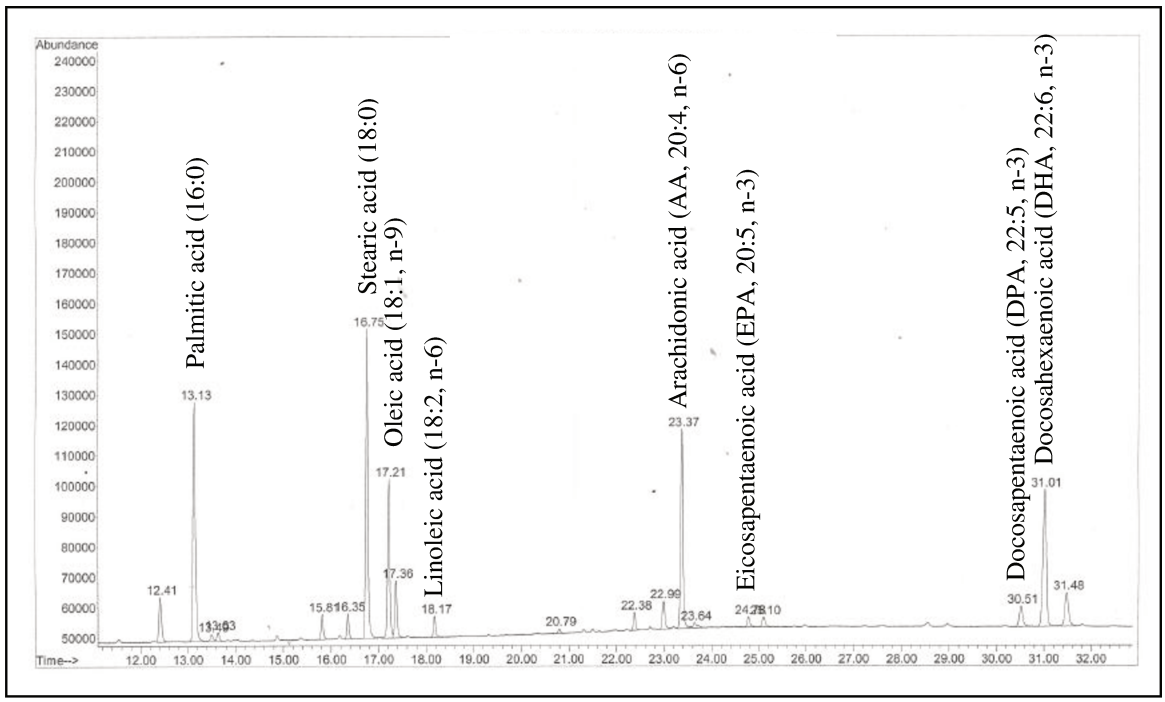

Figure 2.8 Fatty acid profile in 100 μM DHA-Na-treated BMMC. After 24 hr treatment with 100 μM DHA-Na, the fatty acid profile was determined by GC-MS. Data is the representative of three independent experiments with similar results.

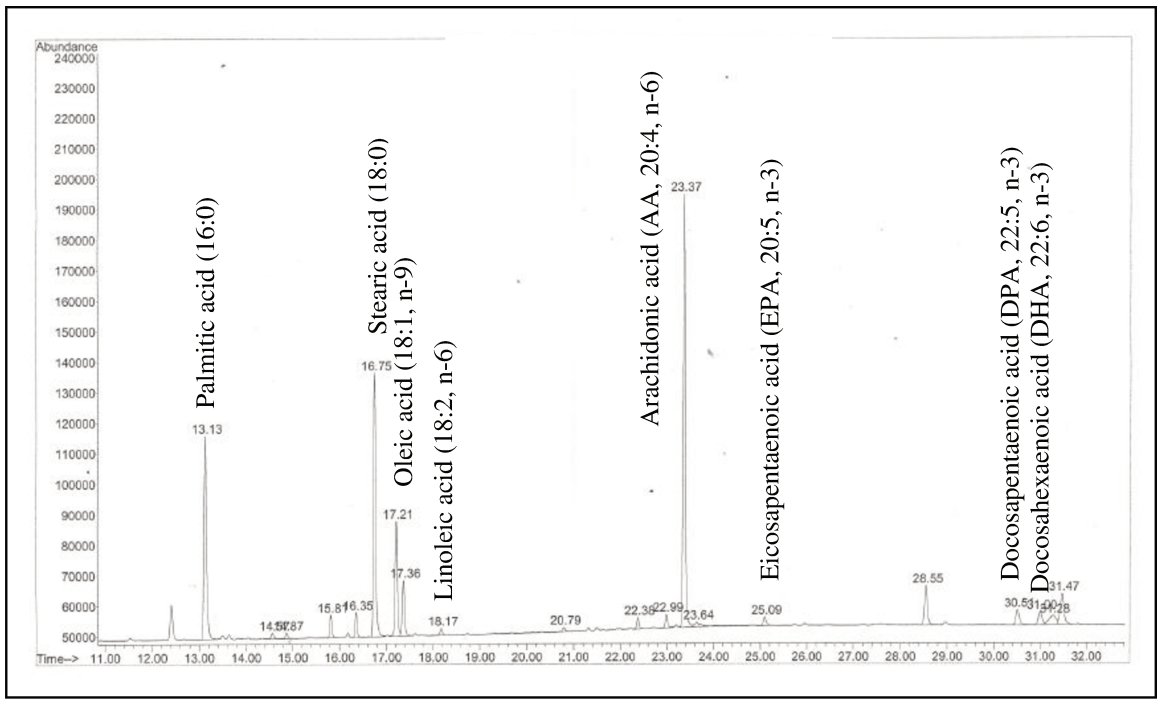

Figure 2.9 Fatty acid profile in 100 µM AA-Na-treated BMMC. After 24 hr treatment with 100 µM AA-Na, the fatty acid profile was determined by GC-MS. Data is the representative of three independent experiments with similar results.

Table 2.1 Fatty acid composition of long chain PUFA-supplemented WT BMMC

Fatty acids	Percentage of total fatty acids (%)			
	Control	EPA-Na	DHA-Na	AA-Na
16:0	15.94 ± 5.65	17.35 ± 6.10	13.44 ± 5.89	14.98 ± 4.68
18:0	25.24 ± 4.41	26.70 ± 2.13	38.12 ± 3.06	24.01 ± 1.17
18:1 n-9	21.16 ± 4.94	14.89 ± 4.50	13.07 ± 0.34	12.41 ± 1.37
18:2 n-6	6.01 ± 1.40	5.34 ± 0.46	3.57 ± 0.87	5.11 ± 0.63
20:4 n-6 (AA)	20.83 ± 3.93	16.72 ± 2.03	14.85 ± 2.97	40.26 ± 4.40**
20:5 n-3 (EPA)	1.00 ± 0.28	6.84 ± 2.56**	0.71 ± 0.04	nd
22:5 n-3 (DPA)	2.57 ± 1.15	9.52 ± 2.13**	1.34 ± 0.86	1.25 ± 0.61
22:6 n-3 (DHA)	7.25 ± 1.25	2.65 ± 1.18	14.91 ± 5.10*	1.99 ± 3.03
n-6/n-3 ratio	2.48 ± 0.08	1.16 ± 0.80*	1.09 ± 0.19*	12.39 ± 0.78**

Values are mean ± SEM, n=3.
*: $p<0.05$, compared with control group, one-way ANOVA followed by the post-hoc Bonferroni test
**: $p<0.01$, compared with control group, one-way ANOVA followed by the post-hoc Bonferroni test
AA: Arachidonic acid; DHA: Docosahexaenoic acid; DPA: Docosapentaenoic acid; EPA: Eicosapentaenoic acid; nd: not detected.

2.4.4 The effect of long chain n-3 PUFA supplementation on mast cell degranulation

EPA-Na supplementation for 24 hr and 48 hr decreased LAD2 degranulation by 30.5% and 35.3% compared to untreated cells (stimulated with 500 ng/mL streptavidin for 30 min) ($p=0.001$ and $p<0.001$). DHA-Na treatment had no effect on LAD2 cell degranulation. AA-Na 24 hr supplementation also inhibited LAD2 degranulation by 55.5% and 56% ($p<0.001$ and $p<0.001$) (Fig. 2.10). EPA-Na and DHA-Na 24 hr treatment decreased BMMC β-hex release activated by DNP-HSA in concentration range of 1-500 ng/mL. In response to 200 ng/mL stimulation, EPA-Na, and DHA-Na supplementation reduced β-hex release by 45.2 % ($p=0.002$) and 45.7% ($p=0.007$) compared to untreated cells. AA-Na supplementation increased β-hex release by 58.1% ($p=0.004$) by cells activated with 1 ng/mL DNP-HSA, and inhibited β-hex release by cells activated with 100 ng/mL (40.1%, $p<0.001$) and 200 ng/mL (34.6%, $p=0.006$) DNP-HSA compared to untreated cells (Fig. 2.11).

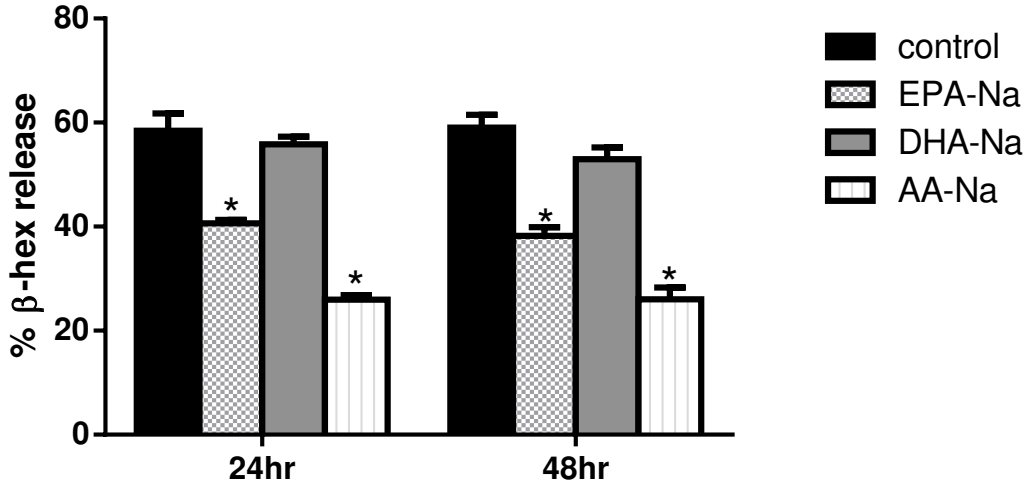

Figure 2.10 IgE/DNP-HSA-induced degranulation of LAD2 cells treated with 100 μM EPA-Na, DHA-Na, and AA-Na for 24 hr and 48 hr. PUFA-Na-treated cells were sensitized with 500 ng/mL IgE-biotin for 24 hr, and then stimulated with 500 ng/mL streptavidin for 30 min. Degranulation was evaluated by measuring β-hex release. Error bars represent SEM (n=3). *: $p<0.01$, compared to untreated control (n=3), one-way ANOVA followed by the post-hoc Bonferroni test.

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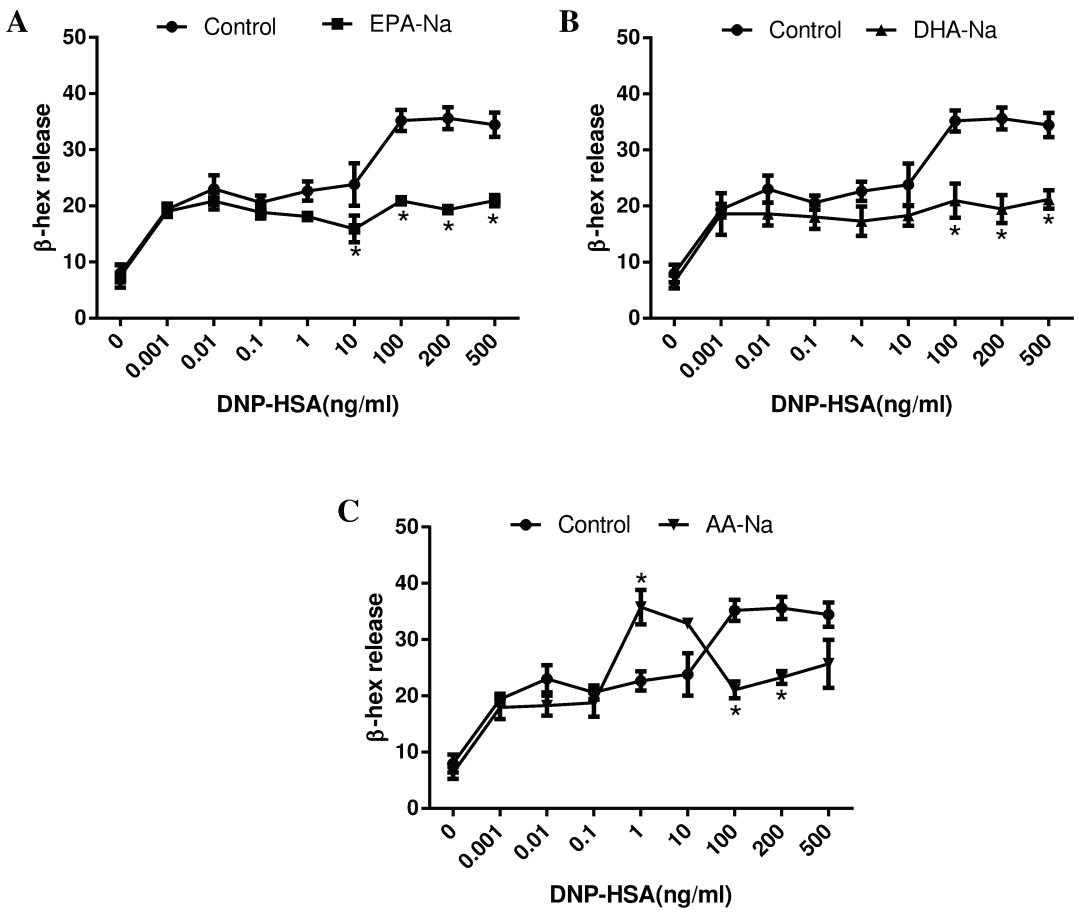

Figure 2.11 IgE/DNP-HSA-induced degranulation of BMMC treated with 100 μM EPA-Na, DHA-Na, and AA-Na for 24 hr. EPA-Na (A), DHA-Na (B), and AA-Na (C)-treated cells were sensitized with 500 ng/mL mouse anti-DNP IgE for 24 hr, and then stimulated with indicated concentrations of DNP-HSA for 30 min. Degranulation was evaluated by measuring β-hex release. Error bars represent SEM (n=3). *: $p<0.05$, compared to untreated control (n=3), two-way ANOVA followed by the post-hoc Bonferroni test.

2.4.5 The effect of long chain n-3 PUFA supplementation on cys-LT release by BMMC

BMMC supplemented with EPA and stimulated with 100 ng/mL DNP-HSA produced significantly less cys-LTs (870.4 pg/mL, p=0.007, Fig. 2.12) compared to the untreated control (1236.8 pg/mL). BMMC supplemented with AA produced more cys-LTs in response to 100 ng/mL DNP-HSA (1623.7 pg/mL, p=0.033) compared to untreated controls (1236.8 pg/mL) (Fig. 2.12). DHA-Na supplementation had no effect on DNP-HSA-induced cys-LT production from BMMC.

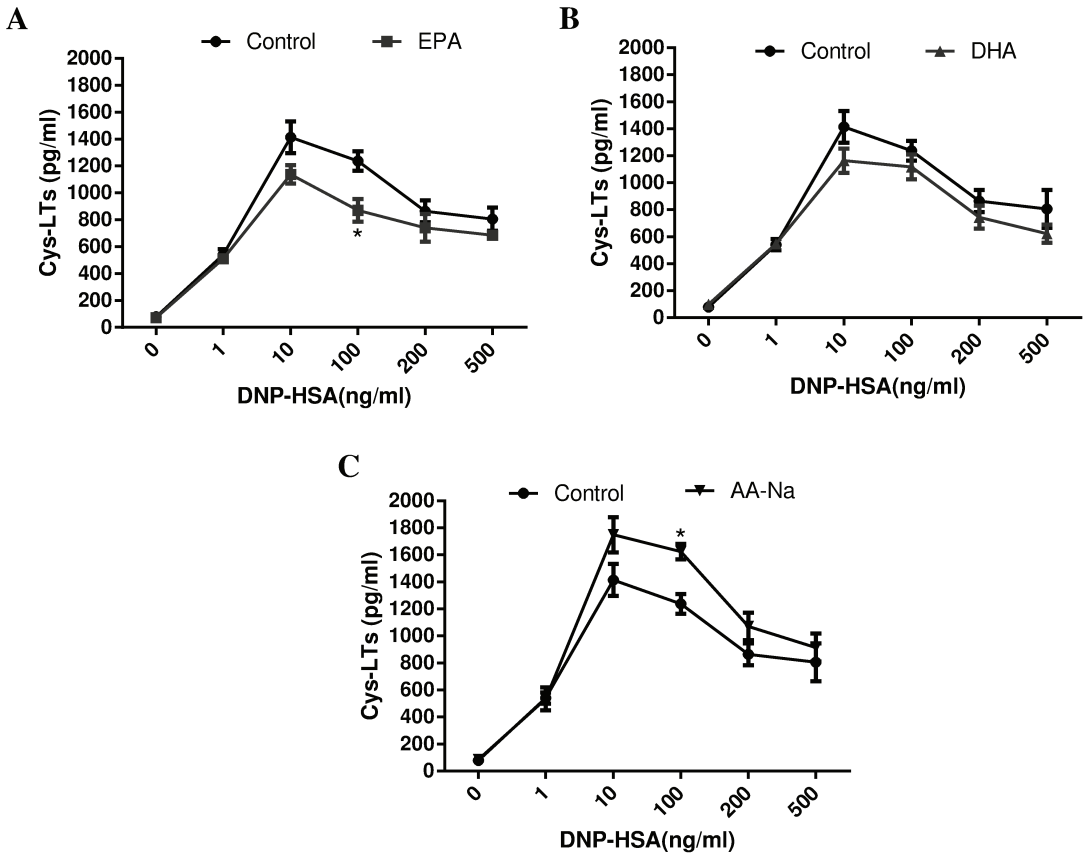

Figure 2.12 IgE/DNP-HSA-induced cys-LT release by BMMC treated with 100 µM EPA-Na, DHA-Na, and AA-Na for 24 hr. EPA-Na (A), DHA-Na (B), and AA-Na (C)-treated cells were sensitized with 500 ng/mL mouse anti-DNP IgE for 24 hr, and then stimulated with indicated concentrations of DNP-HSA for 3 hr. Cys-LT release was evaluated by ELISA. Error bars represent SEM (n=3). *: $p<0.05$, compared to untreated control (n=3), two-way ANOVA followed by the post-hoc Bonferroni test.

2.4.6 The effect of long chain n-3 PUFA supplementation on cytokine/chemokine production by BMMC

In response to 10 ng/mL DNP-HSA, BMMC supplemented with DHA-Na showed less TNF production (1140.1 pg/mL, $p<0.001$) compared to untreated cells (1631.4 pg/mL). DHA supplementation also decreased CCL2 production under 100 ng/mL DNP-HSA (315.8 pg/mL, $p<0.001$) compared to untreated control (437.6 pg/mL). EPA supplementation inhibited CCL2 production (317.5 pg/mL, $p=0.005$) compared to untreated controls (437.6 pg/mL) at 100 ng/mL DNP-HSA stimulation. AA supplementation increased TNF production under 10 ng/mL (3143.8 pg/mL, $p<0.001$) DNP-HSA stimulation compared to untreated control (1631.4 pg/mL) and decrease CCL2 production (320.3 pg/mL, $p=0.045$) at 100 ng/mL DNP-HSA compared to control (437.6 pg/mL) (Fig. 2.13).

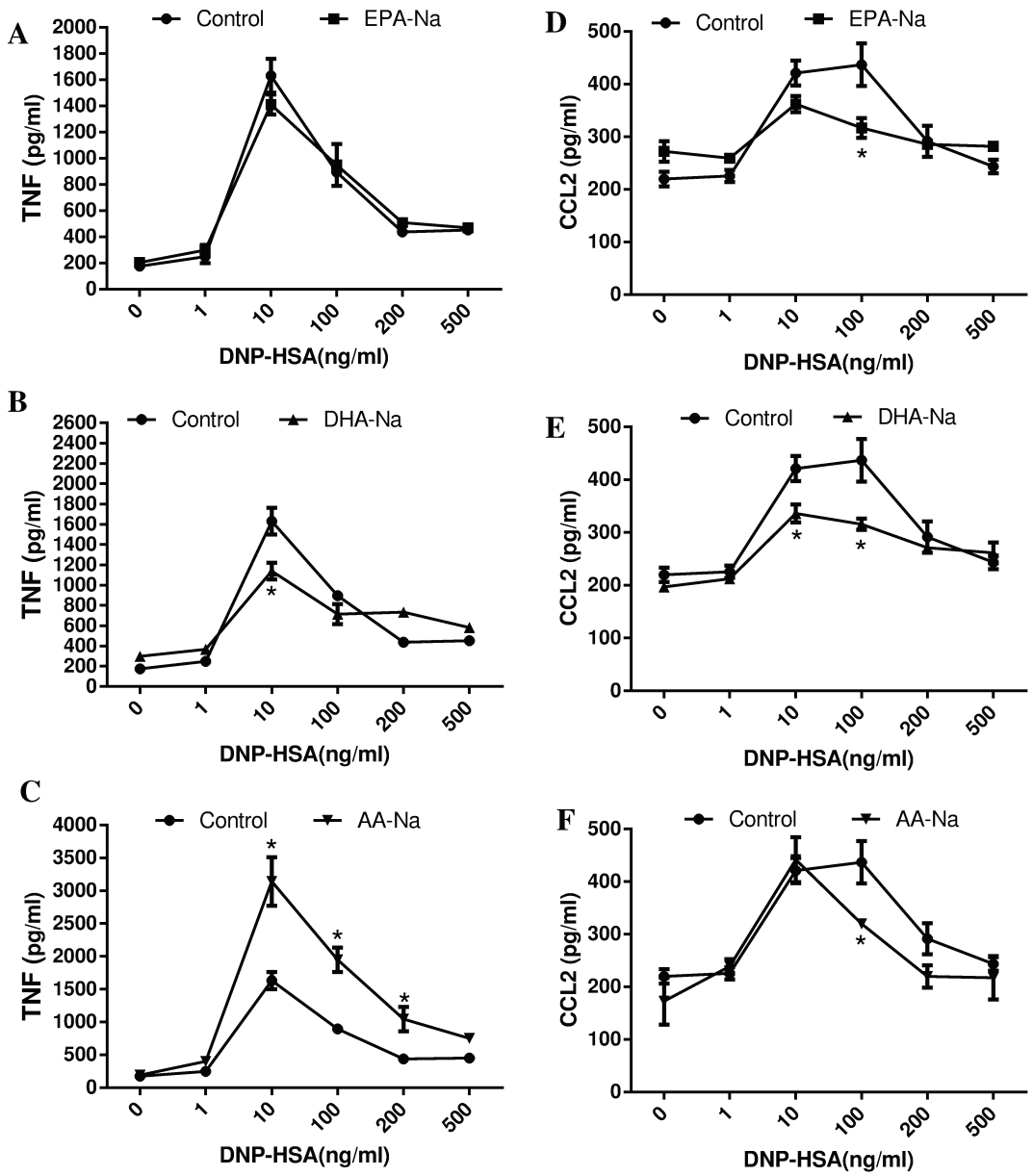

Figure 2.13 IgE/DNP-HSA-induced cytokine/chemokine production by BMMC treated with 100 μM EPA-Na, DHA-Na, AA-Na for 24 hr. After PUFA treatment, cells were sensitized with 500 ng/mL mouse anti-DNP IgE for 24 hr, and then stimulated with indicated concentrations of DNP-HSA for 6 hr. TNF (A, B, and C) and CCL2 (D, E, and F) production was measured by ELISA. Error bars represent SEM (n=3). *: $p<0.05$, compared to untreated control, two-way ANOVA followed by the post-hoc Bonferroni test.

2.4.7 n-3 PUFA levels in fat-1 BMMC

As shown in Fig. 2.14, fat-1 mRNA was expressed in fat-1 BMMC, but not in WT BMMC. In contrast, the housekeeping gene, GAPDH, was equally expressed in both WT and fat-1 BMMC. Among the 8 fatty acids identified with GC-MS (i.e. palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1, n-9), linoleic acid (18:2 n-6), AA (20:4 n-6), EPA (20:5 n-3), docosapentaenoic acid (DPA, 22:5 n-3), and DHA (22:6 n-3)), fat-1 BMMC had significantly lower AA ($p=0.007$) and higher EPA ($p=0.005$) and DPA ($p=0.014$) levels than WT BMMC (Fig. 2.15 and Table 2.2). The n-6/n-3 ratio of WT BMMC was, therefore, significantly higher than that of fat-1 BMMC (6-fold, $p<0.001$, Table 2.2).

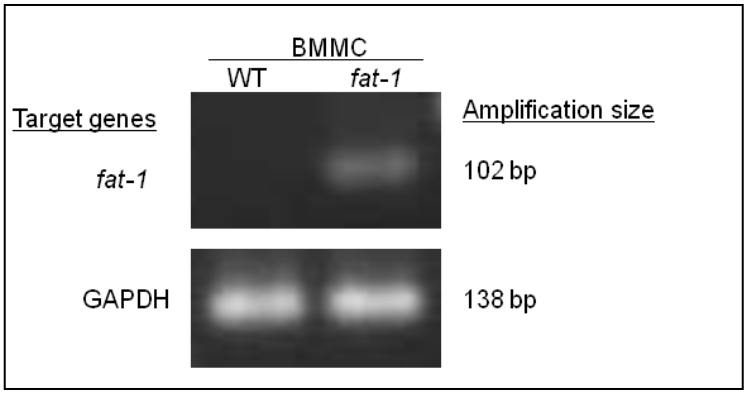

Figure 2.14 mRNA expression of fat-1 (target gene) and GAPDH (housekeeping gene) in WT and fat-1 BMMC. mRNA expression was analyzed by RT-PCR followed with 1.5% agarose gel electrophoresis and detection under UV light. WT: Wild type.

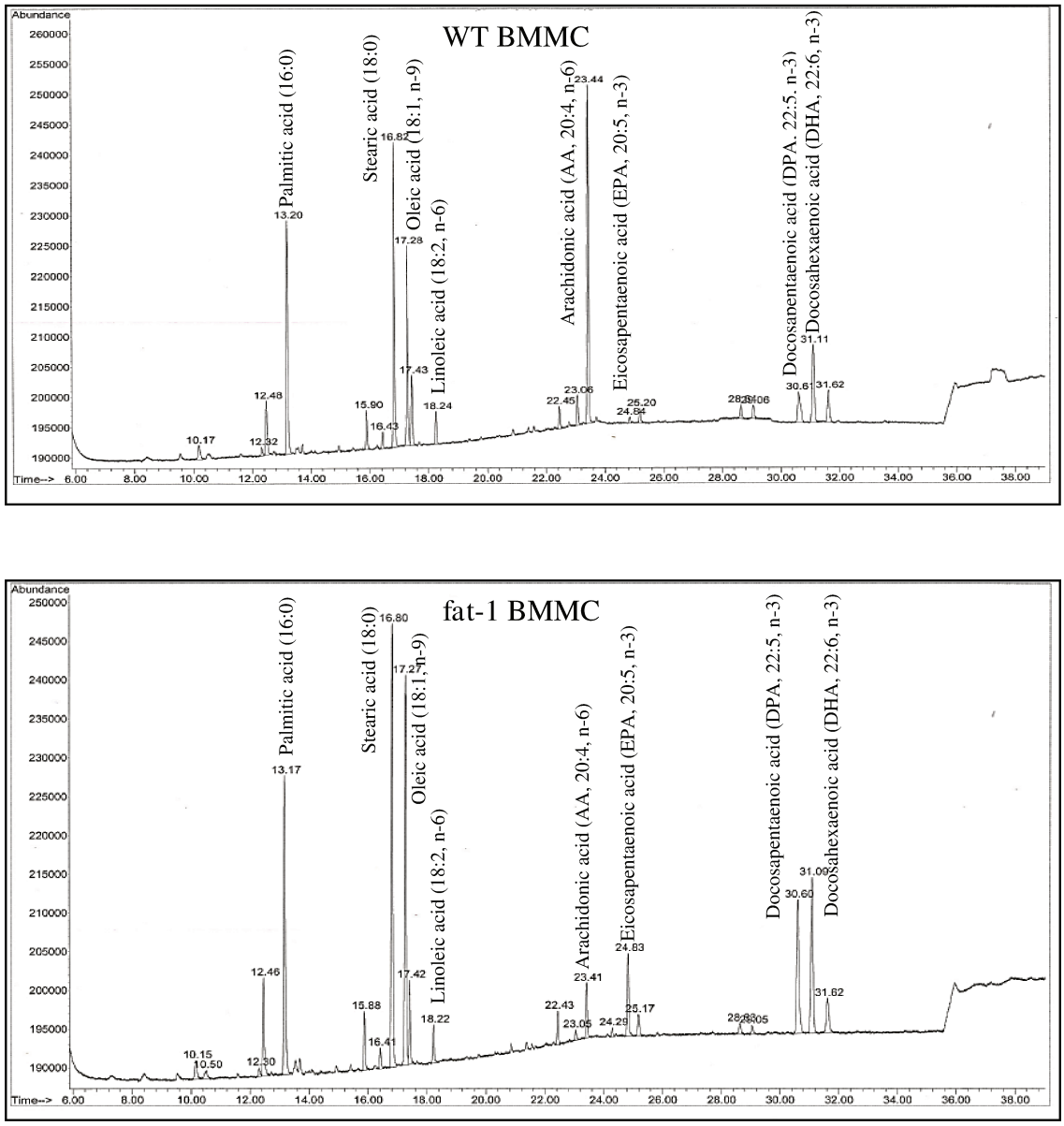

Figure 2.15 Fatty acid composition of WT and fat-1 BMMC. Eight fatty acids were identified using GC-MS, which are marked besides their peaks. WT: Wild type.

Table 2.2 Fatty acid composition of WT and fat-1 BMMC

Fatty acids	Percentage of total fatty acids (%)	
	WT BMMC	fat-1 BMMC
16:0	18.69 ± 4.84	18.38 ± 3.29
18:0	23.42 ± 6.18	22.77 ± 3.08
18:1 n-9	15.04 ± 4.65	20.08 ± 5.31
18:2 n-6	4.23 ± 0.75	5.89 ± 0.82
20:4 n-6 (AA)	24.39 ± 6.62	2.78 ± 0.61**
20:5 n-3 (EPA)	0.59 ± 0.17	5.05 ± 1.09**
22:5 n-3 (DPA)	4.56 ± 1.22	11.93 ± 1.35*
22:6 n-3 (DHA)	9.07 ± 2.76	13.12 ± 2.19
n-6/n-3 ratio	1.84 ± 0.05	0.32 ± 0.05**

Values are mean ± SEM, n=3.
*: $p<0.05$, compared with WT BMMC, Student's t-test.
*: $p<0.01$, compared with WT BMMC, Student's t-test.
AA: Arachidonic acid; DHA: Docosahexaenoic acid; DPA: Docosapentaenoic acid; EPA: Eicosapentaenoic acid; WT: Wild type.

2.4.8 Degranulation, lipid-derived mediator release and cytokine/chemokine production in fat-1 BMMC

Compared to WT BMMC, fat-1 BMMC had lower β-hex release in varying concentrations of DNP-HSA and the difference was significant (29.1% compared to 37.5 % by WT BMMC stimulated with 100 ng/mL DNP-HSA, $p<0.001$) (Fig. 2.16).

Fat-1 BMMC produced less cys-LTs under the stimulation by 10 ng/mL DNP-HSA (1181.3 pg/mL) and 100 ng/mL DNP-HSA (885.2 pg/mL) compared to WT BMMC (1612.7 pg/mL, 10 ng/mL DNP-HSA, $p=0.034$; 1313.1 pg/mL, 100 ng/mL DNP-HSA, $p=0.033$) (Fig. 2.17).

Fat-1 BMMC stimulated via FcεRI produced less TNF compared to WT BMMC (738 pg/mL compared to 982.8 pg/mL in 10 ng/mL DNP-HSA, $p=0.029$; 350.7 pg/mL compared to 707.8 pg/mL in 200 ng/mL DNP-HSA, $p<0.001$) (Fig. 2.18A). Similarly, fat-1 BMMC produced less CCL2 compared to WT BMMC (1131.3 pg/mL compared to 1640.2 pg/mL in 10 ng/mL DNP-HSA, $p<0.001$) (Fig. 2.18B).

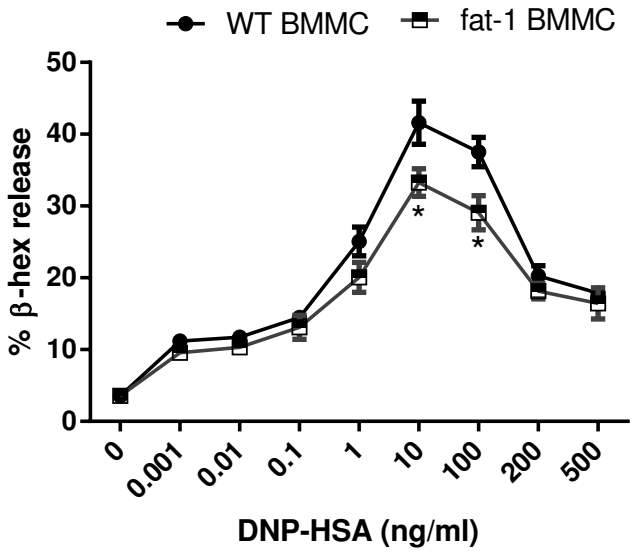

Figure 2.16 IgE/DNP-HSA-induced degranulation of WT and fat-1 BMMC. Cells were sensitized with 500 ng/mL mouse anti-DNP IgE for 24 hr, and then stimulated with indicated concentrations of DNP-HSA for 30 min. Degranulation was evaluated by measuring β-hex release. Error bars represent SEM (n=3). *: $p<0.05$, compared to WT BMMC, two-way ANOVA followed by the post-hoc Bonferroni test. WT: Wild type.

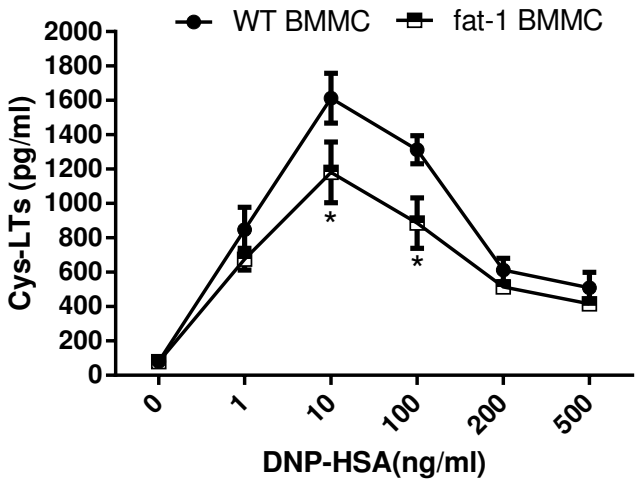

Figure 2.17 IgE/DNP-HSA-induced cys-LT release by WT and fat-1 BMMC. Cells were sensitized with 500 ng/mL mouse anti-DNP IgE for 24 hr, and then stimulated with indicated concentrations of DNP-HSA for 3 hr. Cys-LT release was measured by ELISA. Error bars represent SEM (n=3). *: $p<0.05$, compared to WT BMMC, two-way ANOVA followed by the post-hoc Bonferroni test. WT: Wild type.

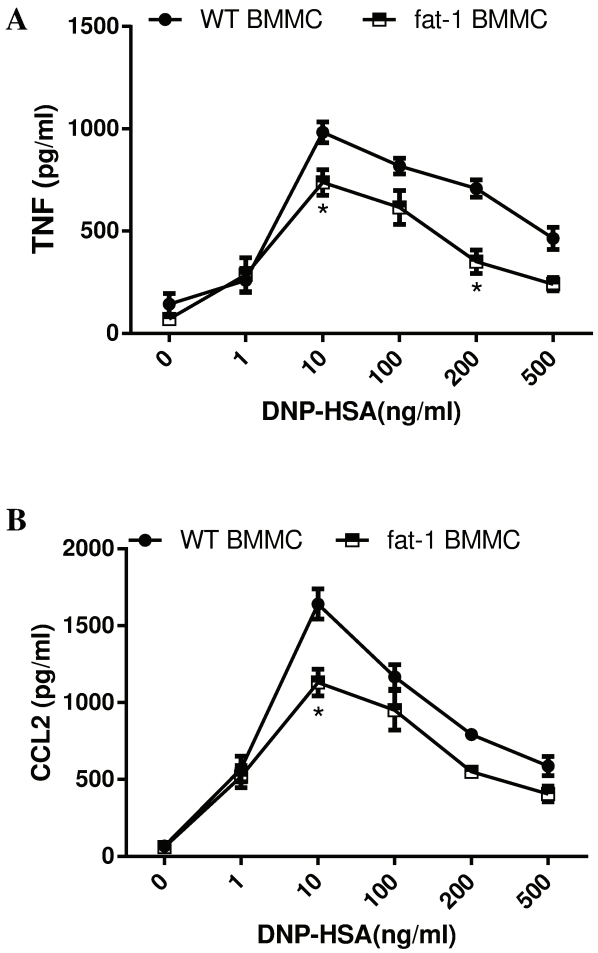

Figure 2.18 IgE/DNP-HSA-induced TNF and CCL2 production by WT and fat-1 BMMC. Cells were sensitized with 500 ng/mL mouse anti-DNP IgE for 24 hr, and then stimulated with indicated concentrations of DNP-HSA for 6 hr. TNF (A) and CCL2 (B) production was measured by ELISA. Error bars represent SEM (n=3). *: $p<0.05$, compared to untreated control, two-way ANOVA followed by the post-hoc Bonferroni test. WT: Wild type.

2.3.9 Calcium release in fat-1 BMMC

Calcium imaging on WT and fat-1 BMMC after IgE sensitization and DNP-HSA stimulation showed that there was no difference in intracellular calcium levels between WT and fat-1 BMMC before activation (Fig. 2.19). However, fat-1 BMMC had lower calcium responses (81%) compared to WT BMMC after cell activation through FcεRI ($p=0.026$).

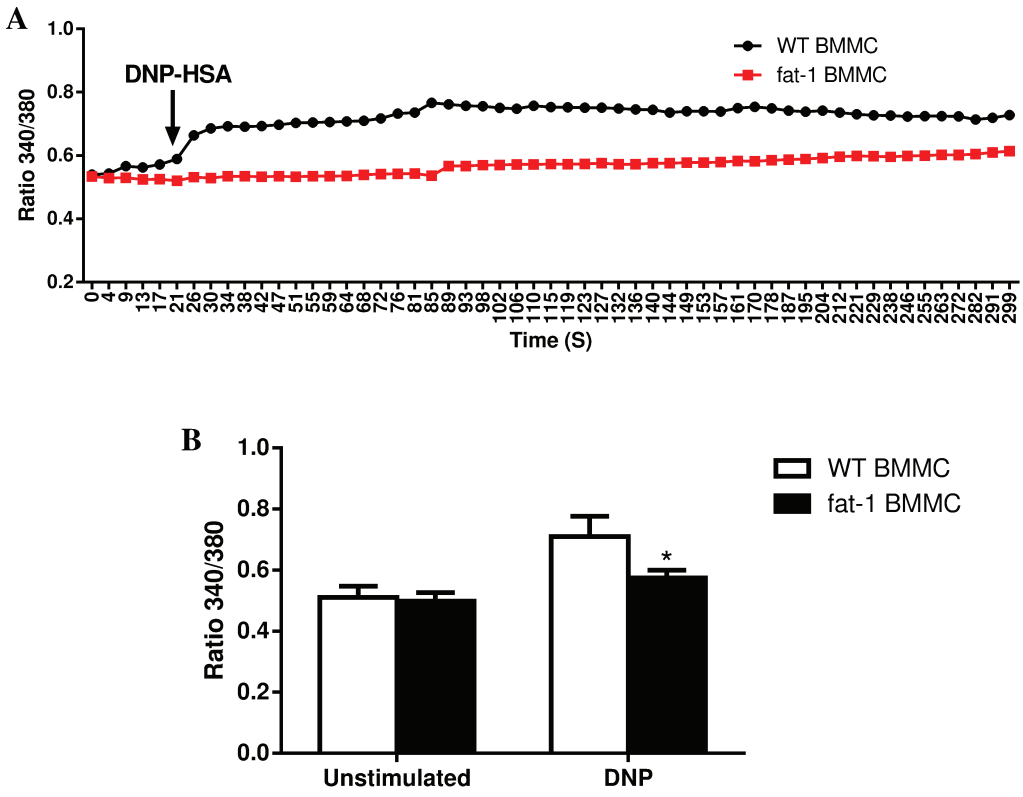

Figure 2.19 IgE/DNP-HSA-induced intracellular calcium mobilization in WT and fat-1 BMMC. (A) Cells were sensitized with 500 ng/mL mouse anti-DNP IgE for 24 hr, and then stimulated with 10 ng/ml DNP-HSA. Intracellular calcium level was evaluated by Fura-2 calcium imaging. (B) Calcium response in 20 randomly chosen cells reported as Mean ± SEM of ratio 340/380 (n=3). *: $p<0.05$, compared with WT BMMC, Student's t-test. WT: Wild type.

2.5 Discussion

In this study, the FcεRI-mediated mediator release of mast cells was clearly shown to be inhibited by either exogenous or endogenous long chain n-3 PUFAs. Exogenous and endogenous introduction of long chain n-3 PUFAs increased n-3 PUFA levels in mast cells and inhibited degranulation, lipid-derived mediator release and cytokine/chemokine production from mast cells activated through FcεRI.

In response to IgE/antigen, mast cells release preformed mediators in seconds, lipid-derived mediators in minutes, and cytokines/chemokines within hours. Many of the mediators play important roles in the process of inflammation. The degranulation process results in a very quick (within seconds) release of preformed mediators to the extracellular environment. These mediators include histamine, heparin, tryptase, chymase, major basic protein and more (Galli et al., 2005). Among them, histamine is one of the best-studied mediators from mast cells that contribute to allergic inflammation both directly and indirectly (MacGlashan, 2003). Mast cells are one of the four well-recognized histamine-producing cells, along with basophils, gastric enterochromaffin-like cells, and histaminergic nerves in the brain (Parsons and Ganellin, 2006). Directly, histamine can increase vascular permeability, cause vasodilation, and induce constriction of smooth muscle cells expressing H_1 receptors. Indirectly, histamine triggers the release of pro-inflammatory cytokines and enhances adhesion molecule expression on T cell, and also triggers pain (Thurmond et al, 2008).

The second group of mast cell mediators, lipid-derived mediators, can be produced from n-3 (EPA) and n-6 PUFAs (AA and dihomo-γ-linolenic acid (DGLA)) (Khanapure et al., 2007). EPA is the precursor of 3-series PGs and 5-series LTs. Whereas AA is the

precursor of 2-series PGs and 4-series LTs (including cys-LTs). DGLA is the precursor of 1-series prostaglandins. However, prostaglandins and leukotrienes derived from EPA have lower pro-inflammatory potential than those from AA. Cys-LTs have a strong effect on smooth muscle contraction, and increased vascular permeability compared to 5-series LTs. Cys-LTs can induce eosinophil migration (Fregonese et al., 2002), and activate eosinophils and mast cells to secret cytokines (Hallstrand and Henderson, 2010). One-series PGs are anti-inflammatory, but the level of DGLA in body is very low (Wang et al., 2012). In HepG2 human hepatic cells, the level of AA is 7-fold higher than the combination of EPA and DHA, and DGLA is not detectable (Yousefi et al., 2012). In mouse liver tissue, the percentage of AA is comparable to the combination of EPA and DHA and 10-fold higher than DGLA (Kelley et al., 2006). New non-classical eicosanoids, protectins and resolvins, were reported to be generated from EPA and DHA, which are all anti-inflammatory (Kohli and Levy, 2009). Furthermore, EPA and AA are using the same enzymes for eicosanoid production, which means competitive inhibition on pro-inflammation eicosanoid production from n-6 PUFAs. So in general, n-3 PUFAs would be considered to be protective against inflammation compared to n-6 PUFAs.

The massive production of cytokines/chemokines requires gene transcription and translation, so are the last mediators to be synthesized and released. TNF and CCL2 are well-documented pro-inflammatory cytokine and chemokine. Some of the key actions of TNF are to trigger the release of pro-inflammatory cytokines/chemokines from macrophages, modulating T cell activation, inducing adhesion molecule expression on endothelial cells, and promoting endothelial cell apoptosis (Zelova and Hosek, 2013).

CCL2 recruits macrophages, T cells and dendritic cells to sites of inflammation, and also activates endothelial cells to produce cytokines such as IL-6, and enhance adhesion molecule expression in endothelial cells, such as intercellular adhesion molecule-1 (ICAM-1) (Conti and DiGioacchino, 2001).

Our data supports earlier observations that long chain n-3 PUFAs have direct effects on mast cell function. In calves, diets enriched in long chain n-3 PUFAs were found to reduce mast cell infiltration in gastrointestinal tract induced by helminthes infection (Muturi et al., 2005). DHA and ALA treatment was reported to decrease tryptase activity, reduce PGE_2 production and histamine release in canine mastocytoma cells activated by the wasp venom peptide, mastoparan (Gueck et al., 2004a; Gueck et al., 2003). ALA decreases histamine release in RBL-2H3 rat basophilic leukemia cells activated by antigen and calcium ionophore A23187 (Kawasaki et al., 1994). In MC/9 mouse mast cells, 6,9,12,15,18,21-tetracosahexaenoic acid (24:6 n-3), EPA and DHA not only decreased the level of LTB_4, LTC_4, 5-hydroxyeicosatetraenoic acid and histamine in resting cells, but also suppressed histamine release from antigen-activated cells (Ishihara et al., 1998). In mouse BMMC, long chain n-3 PUFAs suppressed expression of IL-4, IL-5 and IL-13 at both the gene and protein levels and decreased nuclear expression of GATA-1, 2 (Park et al., 2013). In rat peritoneal mast cells activated with calcium ionophore A23187, and mast cells isolated from tunica mucosa bronchiorum of guinea pig challenged with ovalbumin, long chain n-3 PUFAs are associated with decreased synthesis of PGD_2 and 4-series LTs (Kuwamori et al., 1997; van Haaster et al., 1993). In human mast cell line HMC-1 stimulated with ionomycin/PMA, EPA and DHA incubation decreases production of reactive oxygen

species (ROS) and Th2 cytokines, such as IL-4, IL-13, by suppressing MAPK activation (van den Elsen et al., 2013). In a study using mast cells derived from human umbilical cord mononuclear cells, EPA supplementation showed inhibitory effect on PGD_2 production after challenging cells with IgE/anti-IgE (Obata et al., 1999).

However, these previous studies provided little evidence on the effects of long chain n-3 PUFAs on FcεRI-mediated activation of mast cells and did not address the mechanisms by which long chain n-3 PUFAs modulated mast cell functions. FcεRI mediate the principle signaling pathway through which mast cells are activated during an atopic allergen-driven inflammatory response (Gilfillan and Tkaczyk, 2006). Furthermore, some studies have suggested that long chain n-3 PUFAs may potentiate mast cell mediator synthesis and release. For example, EPA dissolved in ethanol was reported to increase degranulation, TNF release, ROS production, and enhance Syk phosphorylation in RBL-2H3 cells upon anti-TNP IgE/TNP stimulation (Nakano et al., 2005). In C2 canine mastocytoma cells, EPA was reported to increase production of PGE_2 and histamine release after stimulating cells with mastoparan (Gueck et al., 2004b).

In the present study, primary bone marrow-derived mast cells and human LAD2 mast cells were supplemented with long chain n-3 PUFAsfollowed with activation through FcεRI. EPA supplementation reduced degranulation in LAD2 cells and BMMC, inhibited lipid-derived mediator release, and cytokine/chemokine production from BMMC with increased EPA level in the cells. DHA-Na treatment had no effect on degranulation of LAD2 cells and there was no difference between 24 hr and 48 hr treatment of DHA-Na on LAD2 degranulation, which raise the possibility that DHA

may require longer time to have effect on degranulation of LAD2 cells. Incorporation of DHA inhibited degranulation and suppressed cytokine/chemokine production from BMMC, but not cys-LT release, while EPA inhibited cys-LT release from BMMC. The reason is that EPA, instead of DHA, is the precursor for LTs (Calder, 2013). Furthermore, in addition to exogenous supplementation model, a fat-1 transgenic model was used to confirm the results of exogenous model. Fat-1 mouse is a model with unique merits for studies with n-3 PUFAs because the fat-1 mice have a modified n-3/n-6 PUFA profile and the confounding factor of diet which exists in diet intervention studies is diminished. The mice are genetically modified with fat-1 gene from a non-parasitic nematode C. elegans, which encodes an n-3 fatty acid desaturase and enables the animal to endogenously produce n-3 from n-6 PUFAs endogenously (Kang et al., 2004). BMMC generated from fat-1 transgenic mice displayed the expected modified fatty acid profile without any effect on the mast cell phenotype. As expected, fat-1 transgenic BMMC had higher EPA (20:5 n-3), DPA (22:5 n-3), DHA (22:6 n-3, close to be significant) levels and lower AA (20:4 n-6) level, compared to BMMC generated from WT mice. The results showed that fat-1 BMMC had reduced levels of degranulation, lipid-derived mediator synthesis and release, as well as cytokine and chemokine production compared to WT BMMC after IgE/antigen stimulation. The effects may be attributed to higher EPA and DPA levels, and lower AA in fat-1 BMMC. In the present study, endogenous model showed reduced degranulation, cys-LT release and cytokine/chemokine production in fat-1 BMMC. In exogenous model, however, EPA incorporation did not influence TNF production in BMMC. DHA incorporation did not alter cys-LT release in BMMC. The reason might be attributed to the altered n-3 and n-6

PUFA profile in fat-1 BMMC (increase in EPA, DPA and DHA levels, and decease in AA levels). In contrast, inthe exogenous supplementation model, EPA-Na treatment increased EPA and DPA levels, but not DHA and AA levels. DHA-Na treatment only increased DHA level, but not any other PUFAs.

As shown in exogenous model, there is a difference between incorporations of EPA and DHA on mediator release from BMMC. It appears that EPA may be more effective in inhibiting mediator release that does not require gene expression (i.e. preformed and lipid-derived mediators), while DHA is more effective in reducing cytokine/chemokine production in BMMC. This may occur because EPA in contrast to DHA is precursor for PGs and LTs. In addition, with two more carbons and one more double bond, the incorporation of DHA causes an even looser organization of the membrane (higher membrane fluidity) than EPA (Hashimoto, 1999). Furthermore, EPA and DHA supplementation inhibited degranulation in BMMC, but only EPA supplementation decreased degranulation in LAD2 cells, which indicates that different cells have different response to long chain n-3 PUFAs.

The possible mechanisms by which long chain n-3 PUFAs regulate mast cell functions are: 1) Long chain n-3 PUFAs, simply by their incorporation into the membrane, may influence membrane structure and function (Jump, 2002). 2) EPA and DHA are precursors for eicosanoids (PGs, LTs, resolvins) and competitive substrates with AA, and may change the profile of eicosanoid production in body (Tapiero et al., 2002). 3) They may function through the free fatty acid receptors, G-protein coupled receptor (GPR)40 and GPR120 (Briscoe et al, 2003; Oh and Olefsky, 2012). 4) Long chain n-3 PUFAs can regulate gene transcription directly through peroxisome

proliferator-activated receptors (PPARs) (Zuniga et al., 2011). An interesting phenomenon shown in the present study was that AA-Na supplementation also showed inhibitory effects on degranulation (LAD2 cells and BMMC) and CCL2 production (BMMC). Some studies showed that AA can modulate membrane/lipid raft structure and function. Increase membrane fluidity caused by AA supplementation was reported on a broad range of cell types such as macrophages, endothelial cells and neurons (Rietjens et al., 1987; Villacara et al., 1989; Yang et al., 2011). AA supplementation was shown to increase the AA content of both rafts and non-raft regions in C2 mast cells (Basiouni et al., 2012).

In summary, the current study provided evidence for inhibitory effects of long chain n-3 PUFAs on mediator release from mast cells activated through the FcεRI signaling pathway. Exogenous supplementation and endogenous production of n-3 PUFAs have similar effects on mast cell activation – even though endogenous production of n-3 PUFAs results in a greater alteration on fatty acid profile. EPA may be more potent on inhibiting mediator release that do not require gene expression (preformed and lipid-derived mediators), while DHA is more affective on suppressing cytokine/chemokine production.

2.6 References

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CHAPTER 3

LONG CHAIN N-3 POLYUNSATURATED FATTY ACIDS INHIBIT FcεRI-MEDIATED SIGNAL TRANSDUCTION BY ALTERING EXPRESSION OF SIGNALING MOLECULES AND DISRUPTING FcεRI ASSOCIATION WITH LIPID RAFTS

3.1 Abstract

Aim of the study: n-3 polyunsaturated fatty acids (PUFAs) were shown to inhibit mediator release from mast cells activated through Fc ε receptor I (FcεRI). This study is to evaluate how long chain n-3 PUFAs alter FcεRI-mediated signal transduction.

Methodology: The total expression and phosphorylation of Lyn, spleen tyrosine kinase (Syk) and linker of activated T cells (LAT) in wild type (WT) and fat-1 bone marrow-derived mast cells (BMMC) before and after activation was measured by western blot analysis. FcεRI cell surface expression in BMMC and laboratory of allergic diseases 2 (LAD2) cells were evaluated by flow cytometry. Lipid rafts were isolated by sucrose gradient centrifugation. Fatty acid profile in lipid rafts was measured by GC-MS. FcεRI expression in whole cell and lipid raft were determined by western blot analysis.

Results: The phosphorylation of Lyn, Syk and LAT, and total expression of Lyn and LAT were decreased in fat-1 BMMC. The cell surface and whole cell expression of FcεRI were not changed in fat-1 BMMC and long chain n-3 PUFA-treated BMMC and LAD2 cells. However, FcεRI expression in lipid rafts was decreased in fat-1 BMMC and mast cells supplemented with long chain n-3 PUFAs. After eicosapentaenoic acid (EPA) supplementation, the expression of FcεRI in lipid rafts of both resting and activated BMMC was reduced compared to control. long chain n-3 PUFA levels were increased in lipid rafts of fat-1 BMMC compared to WT.

Conclusion: Long chain n-3 PUFAs inhibit FcεRI-mediated signal transduction in mast cells, by reducing the expression of Lyn and LAT and disrupting FcεRI association with lipid rafts through altering lipid raftfatty acid profile.

3.2 Introduction

In chapter 2, n-3 polyunsaturated fatty acids (PUFAs) were shown to inhibit Fc ε receptor I (FcεRI)-mediated mast cell degranulation and lipid-derived mediator and cytokine/chemokine production and release, both exogenously and endogenously, which suggests FcεRI-mediated signal transduction was inhibited by long chain n-3 PUFAs.

FcεRI is expressed on mast cells as a tetramer, which contains one α chain for immunoglobulin E (IgE) binding, one β and two γ chains for signal transduction using the immunoreceptor tyrosine-based activation motifs (ITAMs) (Fig. 1.1). The cross-linking of IgE-bound FcεRI with multivalent antigen results in the phosphorylation of ITAMs by Lyn kinase. Phosphorylated ITAMs recruit and activate spleen tyrosine kinase (Syk) (Johnson et al., 1995), which activates protein kinase C (PKC). PKC mediates degranulation and the activation of transcription factor, nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB). Syk also phosphorylates the linker of activated T cells (LAT), which is an adaptor protein mediating the activation of phospholipase C-γ (PLC-γ) (Siraganian et al., 2010). PLC-γ generates diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP3) from phosphatidylinositol 4,5-bisphosphate (PIP2), which activates PKC (Teixeira et al., 2003) and releases calcium from the endoplasmic reticulum (ER) (Taylor and Thorn, 2001). Another adaptor protein, growth factor receptor-bound protein 2 (Grb2), is also activated by phosphorylated LAT, which mediates the activation of mitogen-activated protein kinase (MAPK) pathways and activator protein 1 (AP-1) transcription factor activation (Fig. 1.3). Cytosolic phospholipases A2 (cPLA2) can be activated by ERK, which causes the release of eicosapentaenoic acid (EPA, n-3) and arachidonic acid (AA, n-6) from

membranes (Fujishima et al., 1999). Cyclooxygenases (COX) and lipoxygenases (LOX) act on EPA and AA to produce eicosanoids (Vane, 1971).

Thus, FcεRI-mediated signal transduction requires precise interactions with membrane-proximal second messengers, thought to occur on discrete cholesterol-rich microdomains termed as lipid rafts (Simons and Toomre, 2000; Siraganian et al., 2010). Lipid rafts function as platforms for receptor-ligand binding and cross-linking and are distinct from non-raft membrane regions because they contain high levels of cholesterol, sphingolipids, transmembrane proteins, acylated proteins, and glycophosphatidylinositol (GPI)-anchored proteins. The content of saturated fatty acids is thought to be higher in lipid rafts compared to other regions (Lingwood and Simons, 2010). The special composition of lipid rafts causes the rafts to be more tightly packed, and results in a liquid-ordered state, allowing them to facilitate incredibly rapid signaling events. Lipid rafts are thought to have concentrated molecules required for signaling and a more stable membrane local environment, thus making them an ideal platform for FcεRI-mediated signal transduction (Barua and Goldstein, 2012; Lingwood and Simons, 2010), because the signal transduction mediated by FcεRI is incredibly quick. The preformed mediators can be released within seconds.

n-3 PUFAs are components of phospholipids and sphingolipids, the two main forms of membrane lipids. Since unsaturated fatty acyl chains have a less tendency to pack together, n-3 PUFAs may alter raft property and function, thus modifying the cell's response to receptor-mediated stimuli. Recently, it has been suggested that changes in the lipid component of lipid rafts can disrupt the signal transduction mediated by some receptors. In human T cells, docosapentaenoic acid (DHA) was shown to inhibit IL-2

receptor signal transduction by increasing n-3 PUFA levels and decreasing n-6/n-3 ratio in lipid rafts. Signal transducer and activator of transcription (STAT)5a and STAT5b recruitment to lipid rafts were inhibited as a result of reduced IL-2 receptor expression in lipid rafts (Li et al., 2005). In human breast cancer cells, eicosapentaenoic acid (EPA) and DHA treatments inhibit epidermal growth factor receptor (EGFR) expression in lipid rafts and EGFR signal transduction by decreasing levels of sphingomyelin, cholesterol, and DAG in lipid rafts (Schley et al., 2007).

Long chain n-3 PUFA supplementation increases n-3 PUFA levels in both raft and non-raft regions of membranes from canine mastocytoma cells (Basiouni et al., 2012). Thus, we hypothesized that long chain n-3 PUFAs alter lipid raft propertyin mast cells and modify the dynamic partitioning of FcεRI, thereby inhibit the signaling events initiated by FcεRI cross-linking. The expression of some signaling proteins may also be influenced by long chain n-3 PUFAs, because long chain n-3 PUFAs may bind to free fatty acid receptors and peroxisome proliferator-activated receptors (PPARs) to modulate gene transcription (Calder, 2012). To test this hypothesis, we used two models of long chain n-3 PUFA incorporation: 1) Long chain n-3 PUFAs were exogenously supplemented to laboratory of allergic diseases 2 (LAD2) cells and wild type (WT) bone marrow-derived mast cell (BMMC) in culture; 2) BMMC were cultivated from bone marrows of fat-1 transgenic mice, which have enhanced long chain n-3 PUFA levels and decreased n-6 PUFA levels endogenously. The effect of long chain n-3 PUFAs on FcεRI-mediated signal transduction in mast cells was examined in the context of FcεRI distribution and mobilization in lipid rafts and early phosphorylation events.

3.3 Materials and Methods

3.3.1 Growth of mouse BMMC and human LAD2 cells

The method is the same as described in chapter 2, section 2.3.2.

3.3.2 Fatty acid supplementation to BMMC and LAD2 cells

The method is the same as described in chapter 2, section 2.3.5.

3.3.3 Flow cytometry

The method is the same as described in chapter 2, section 2.3.4. Mean fluorescence intensity (MFI), which represents the shift of fluorescence intensity in the cell population measured, was generated using WinMDI 2.9 software.

3.3.4 Lipid raft isolation

Lipid rafts were isolated by sucrose gradient centrifugation modified from Brown (Brown, 2002). Two hundred million cells were collected and washed with ice-cold Tris/NaCl/EDTA (TNE) buffer. Cells were then lysed on ice with 1% Triton X-100 dissolved in TNE/P buffer (TNE supplemented with 0.2 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, Oakville, ON), 1μg/ml leupeptin (Sigma-Aldrich), and 1μg/ml pepstatin (Sigma-Aldrich)). Cell lysate was mixed with ice-cold TNE/P buffer containing 80% (w/v) sucrose before transferring to a 14 × 89 mm ultracentrifuge tube (Beckman Coulter, Mississauga, ON). Cell lysate/80% sucrose mixture was then overlaid by ice-cold TNE/P buffer containing 35% (w/v) sucrose which was covered to the top by ice-cold 5% (w/v) sucrose in TNE/P buffer (Fig. 3.1A). After centrifugation with a SW-41 rotor (Beckman Coulter) for 3 hours (hr) at 100, 000× g at 4°C, a visible

band of lipid raft fraction was harvested at the 5%/35% sucrose interface (Fig. 3.1B). The lipid raft fraction was then transferred to a new ultracentrifuge tube, diluted with ice-cold TNE/P buffer, and centrifuged for 1 hr at 100, 000× g at 4°C. After removing the supernatant, the pellet was re-suspended in 100 to 200 µl TNE/P buffer and centrifuged for 10 minutes (min) at 14, 000 rpm at 4°C. The supernatant was discarded and the pellet dissolved directly in 2× SDS sample buffer (Life Technologies, Burlington, ON), prior to boiling for 5 min.

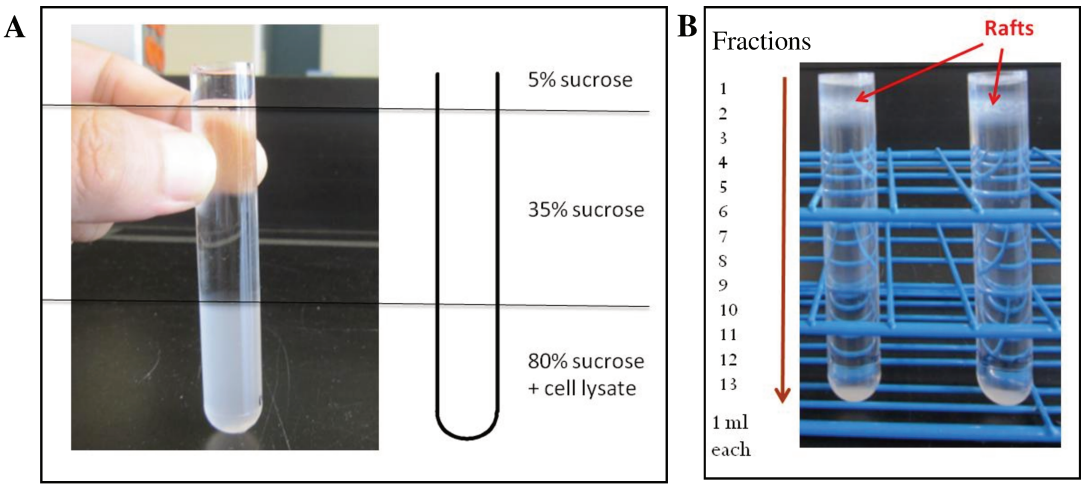

Figure 3.1 Lipid raft isolation using sucrose gradient centrifugation. (A) Sucrose gradient before centrifugation. (B) Sucrose gradient after centrifugation. After sucrose gradient centrifugation, 13 fractions, 1 mL each, can be collected from top to the bottom for lipid raft identification using western blot analysis.

3.3.5 Gas chromatography-mass spectrometry (GC-MS) of lipid rafts

The fatty acid profile of lipid rafts was measured by GC-MS using method described in chapter 2, section 2.3.8.

3.3.6 Lipid raft identification and FcεRI distribution

For lipid raft identification, thirteen fractions were collected from the top of the gradient tube with 1 ml from each lysate fractionation (Fig. 3.1) by sucrose gradient centrifugation as mentioned above. After boiling in 2× SDS sample buffer (Life Technologies) for 5 min, lipid raft marker Lyn expression in each fraction was evaluated by western blot analysis.

For FcεRI distribution measurement, EPA-Na-treated cells were sensitized with 500 ng/mL anti-2,4-dinitrophenyl (DNP) IgE (Sigma-Aldrich) for 24 hr, then activated with 10 ng/mL DNP-human serum albumin (HSA) (Sigma-Aldrich) for 5 min. After cell lysate fractionation by sucrose gradient centrifugation, FcεRI expression in 13 fractions was evaluated by western blot analysis.

3.3.7 Protein isolation from BMMC and LAD2 cells

Protein was extracted from cells by lysing the cells with lysis buffer containing 1% Triton X-100, 1× complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN), 1× protease inhibitor cocktail (Sigma-Aldrich), 50 μg/ml 3,4 dichloroisocoumarin (Roche Molecular Biochemicals), 1 mM benzamidine (Sigma-Aldrich), 1 mM sodium orthovanadate (Sigma-Aldrich), 5.4 mM sodium pyrophosphate (Sigma-Aldrich), and 50 mM sodium fluoride (Sigma-Aldrich) in tris-buffered saline (TBS). After measuring the protein concentration using the

Bradford protein assay (Sigma-Aldrich) (Bradford, 1976), the protein concentration for each sample was adjusted to the same level with lysis buffer. Finally, samples were boiled for 5 min in 4× NuPage sample buffer (Life Technologies).

3.3.8 Western blot analysis

Whole cell protein, lipid raft, and gradient fraction samples were electrophoresed on a NuPage Novex 4-12% Bis-Tris Gel (Life Technologies) at 200 V for 30 min. Then, the separated proteins were transferred to a nitrocellulose membrane (Life Technologies) at 30V for 1 hr. Membranes were then removed and washed with TBST (TBS containing 0.05% tween-20). After blocking with 5% nonfat milk in TBST at 4°C overnight, membrane was probed with rabbit polyclonal anti-mouse Lyn, pho-Lyn, Syk, pho-Syk, LAT and Pho-LAT antibodies (Cell Signaling Technology, Danvers, Massachusetts), rabbit polyclonal anti-mouse and anti-human FcεRIα antibodies (Santa Cruz Biotechnology, Santa Cruz, California) and mouse monoclonal anti-actin antibody (Sigma-Aldrich) diluted with 5% nonfat milk in TBST at room temperature for 3 h. Membranes were then washed with TBST three times for 15 min each and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) and HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) diluted with 5% nonfat milk in TBST at room temperature for 1 hr. After washing with TBST three times for 15 min each, the blot was developed in chemiluminescent peroxidase substrate (Sigma-Aldrich) for 1 min and exposed to ultraviolet (UV) light in a ChemiDoc XRS system (BD Biosciences, Mississauga, ON) for image acquisition.

3.3.9 Statistical analysis

The differences in protein expression and fatty acid profile between WT and fat-1 BMMC were determined using Student's t-test. The differences between the PUFA-treated cells were evaluated by one-way analysis of variance (ANOVA) followed by the post-hoc Bonferroni test. Two-way ANOVA followed by the post-hoc Bonferroni test was used to determine the difference of expression and phosphorylation of signaling molecules at different time points between WT and fat-1 BMMCs, and of the distribution of FcεRI in different fractions. Statistical significance was set at $p<0.05$. Statistical analysis was performed using SPSS 11.5 statistical software package (IBM Corporation, Armonk, NY).

3.4 Results

3.4.1 The effects of long chain n-3 PUFAs on FcεRI-mediated signal transduction in BMMC

IgE/antigen induced a significant increase in phosphorylation of Lyn, Syk, and LAT after 5 min which reached a maximum at 10-25 min. Fat-1 BMMC had lower phosphorylation of Lyn, Syk, LAT compared to WT BMMC after DNP-HSA stimulation. For example, after 10 min DNP-HSA activation, the phosphorylation of Lyn, Syk and LAT in fat-1 BMMC were 28% ($p=0.044$), 30.1% ($p=0.035$) and 33% ($p=0.011$), respectively, lower than in WT BMMC (Fig. 3.2). Fat-1 BMMC expressed lower amounts of Lyn and LAT. In unstimulated cells, the expression of total Lyn and LAT in fat-1 BMMC were 29.8% ($p=0.006$) and 33.9% ($p=0.031$), respectively, lower than in WT BMMC (Fig. 3.2).

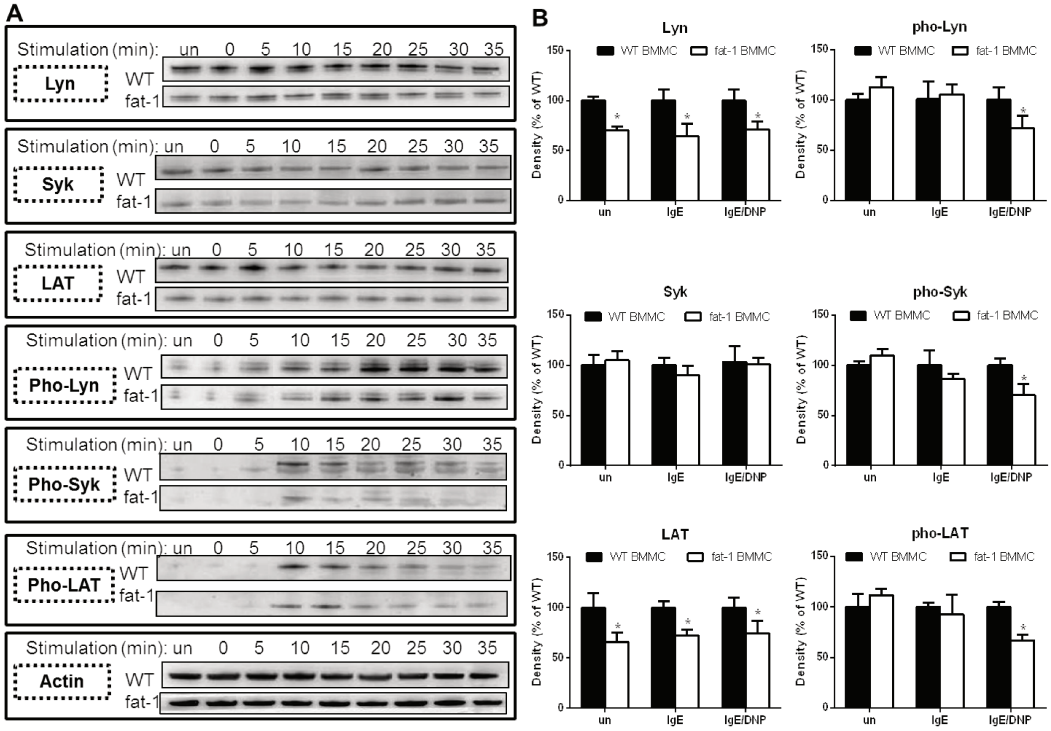

Figure 3.2 The expression and phosphorylation of Lyn, Syk, LAT in WT and fat-1 BMMC activated with IgE/DNP-HSA. BMMC were first sensitized with anti-DNP IgE for 24 hr and then activated with 10 ng/mL DNP-HSA for the indicated time points. (A) Western blot measurement of total expression and phosphorylation of Lyn, Syk, LAT with actin as loading control. Data are the representative of three independent experiments with similar results. (B) The relative density (% of WT) of total and phosphorylated Lyn, Syk, and LAT in fat-1 BMMC after activating IgE-sensitized cells with 10 ng/mL DNP-HSA for 10 min. Error bars represent standard error of the mean (SEM) (n=3). *: $p<0.05$, compared to WT BMMC, two-way ANOVA followed by the post-hoc Bonferroni test. un: untreated; WT: Wild type.

3.4.2 The effects of long chain n-3 PUFAs on FcεRI expression on the cell surface of mast cells

WT BMMC and fat-1 BMMC expressed comparable levels of FcεRI on the cell surface (Fig. 3.3). In addition, EPA-Na, DHA-Na, AA-Na supplementation did not alter FcεRI cell surface expression in BMMC and LAD2 cells (Fig. 3.4 and 3.5).

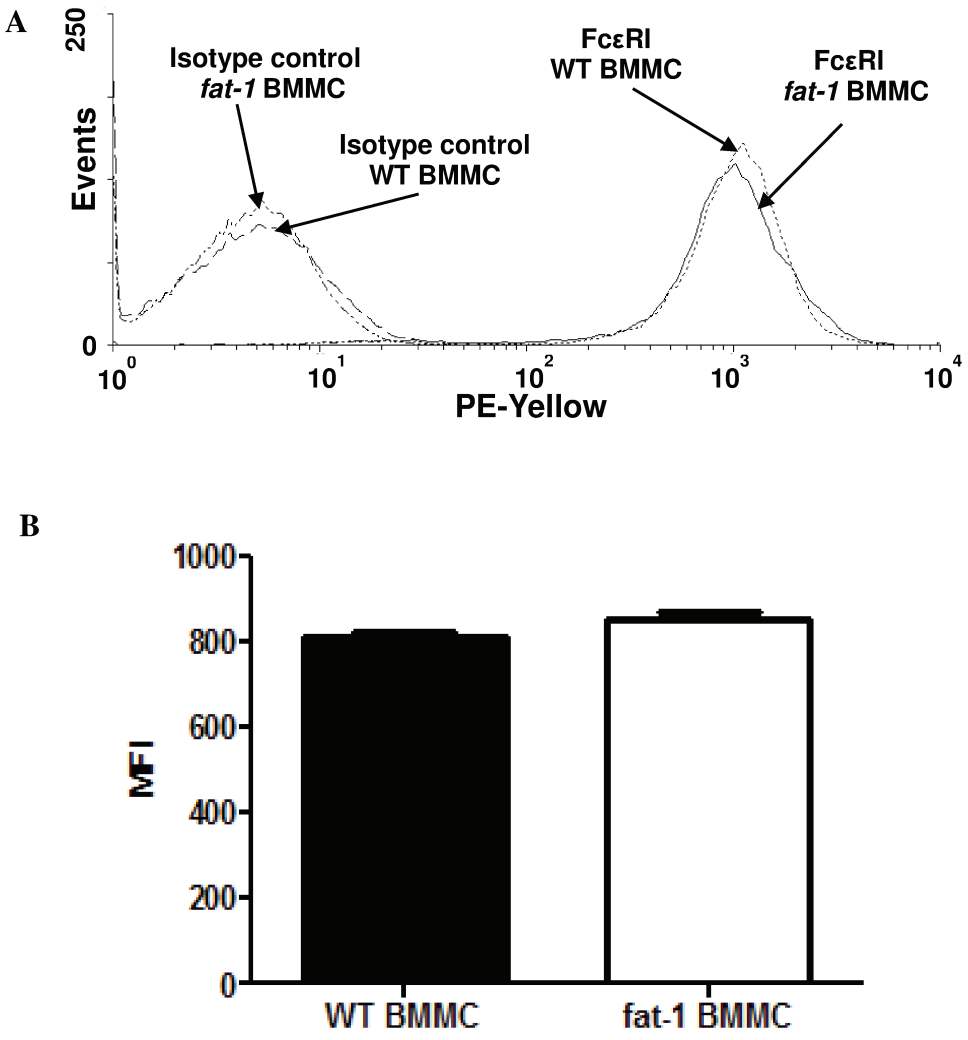

Figure 3.3 FcεRI expressions on the cell surface of WT and fat-1 BMMC. The cell surface expression of FcεRI on WT and fat-1 BMMC was measured by flow cytometry. (A) Histogram of flow cytometry analysis of cell surface expression of FcεRI and isotype control on WT and fat-1 BMMC. (B) The MFI of cell surface FcεRI on WT and fat-1 BMMC. Error bars represent standard error of the mean (SEM) (n=3). MFI: Mean fluorescence intensity; WT: Wild type.

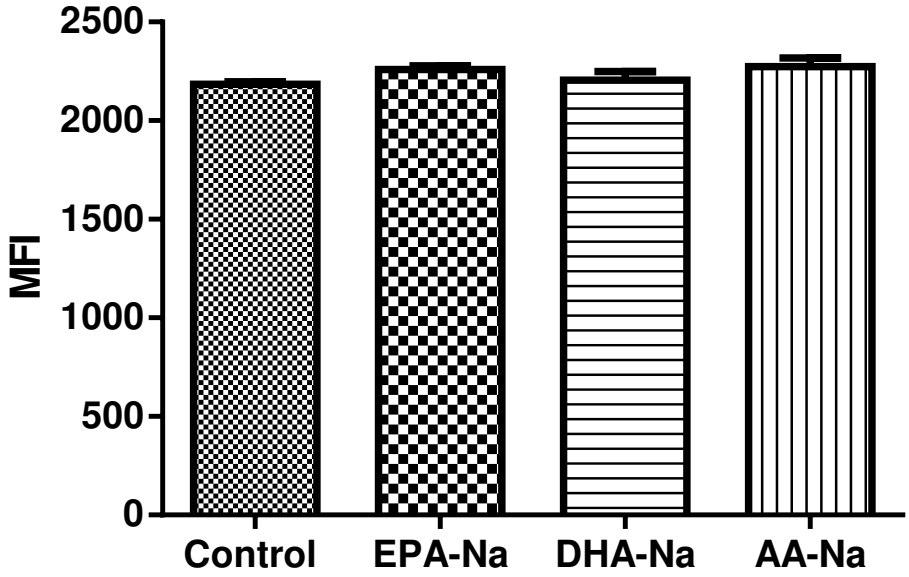

Figure 3.4 FcεRI expressions on the cell surface of long chain PUFA-supplemented WT BMMC. WT BMMC were first treated with 100 μM EPA-Na, DHA-Na, and AA-Na for 24 hr. The MFI of cell surface FcεRI was then measured by flow cytometry and is compared to untreated control. Error bars represent SEM (n=3). MFI: Mean fluorescence intensity.

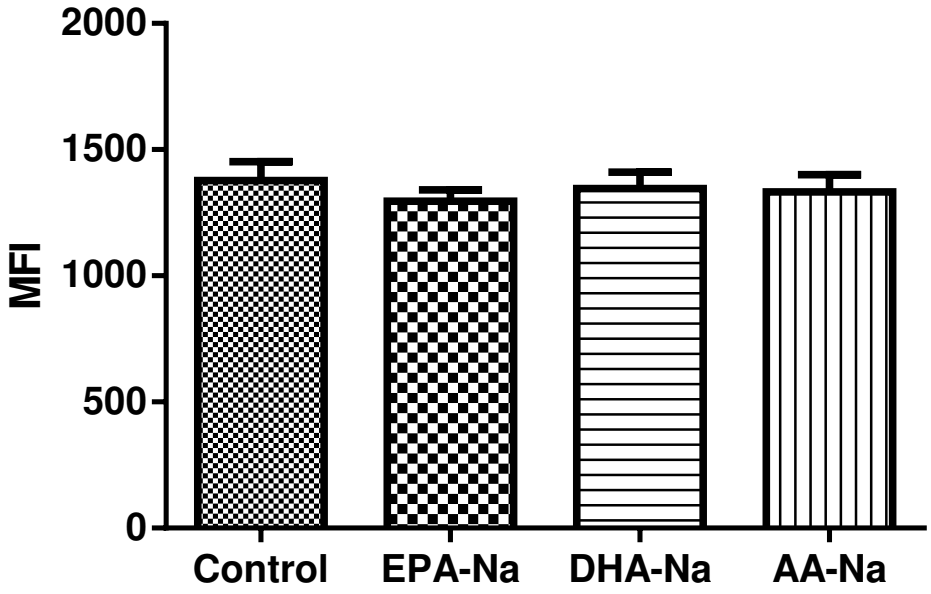

Figure 3.5 FcεRI expressions on the cell surface of long chain PUFA-supplemented LAD2 cells. LAD2 cells were first treated with 100 μM EPA-Na, DHA-Na, and AA-Na for 24 hr. The MFI of cell surface FcεRI was then measured by flow cytometry and is compared to untreated control. Error bars represent SEM (n=3). MFI: Mean fluorescence intensity.

3.4.3 The effects of long chain n-3 PUFAs on FcεRI expression in whole mast cells

There was no difference in whole cell FcεRI expression between WT and fat-1 BMMC (Fig. 3.6). In addition, EPA and DHA supplementation did not change whole cell FcεRI expression in BMMC. However, AA supplementation increased whole cell FcεRI expression (84.1%, p=0.002, Fig. 3.7). In human mast cells LAD2 cells, EPA, DHA, and AA supplementation all had no effect on FcεRI expression in whole cells (Fig. 3.8).

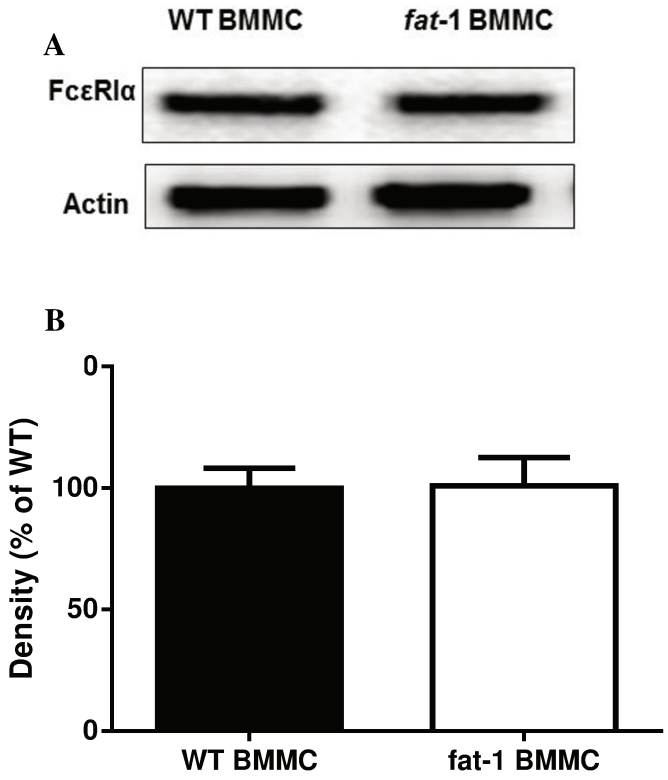

Figure 3.6 FcεRI expressions in whole cell lysates of WT and fat-1 BMMC. (A) Western blot evaluation of FcεRI expression in whole cell lysates of WT and fat-1 BMMC with actin as the loading control. Data are the representative of three independent experiments with similar results. (B) The relative density of whole cell FcεRI (% of wild type) in fat-1 BMMC. Error bars represent SEM (n=3). WT: Wild type.

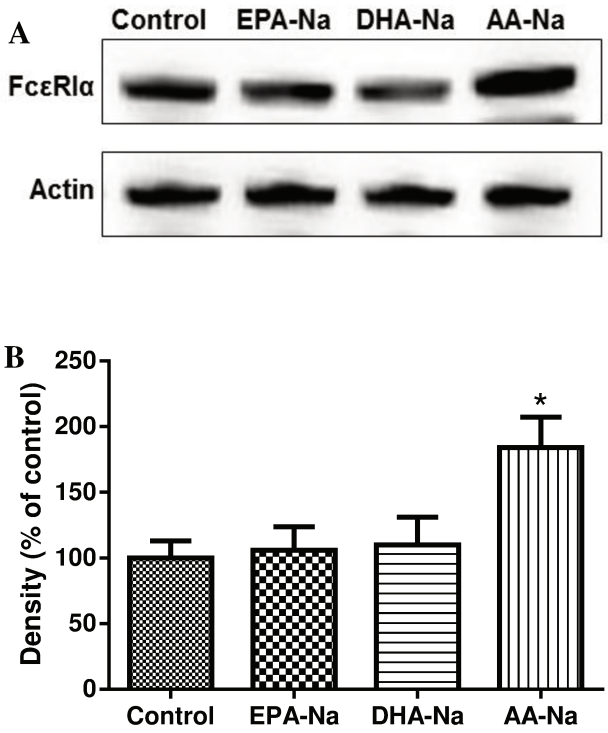

Figure 3.7 FcεRI expressions in whole cell lysates of long chain PUFA-supplemented WT BMMC. WT BMMC were first treated with 100 μM EPA-Na, DHA-Na, and AA-Na for 24 hr. The whole cell expression of FcεRI was measured by western blot analysis. (A) Western blot determination of FcεRI expression in whole cell lysates of PUFA-treated BMMC with actin as the loading control. Data are the representative of three independent experiments with similar results. (B) The relative density of whole cell FcεRI (% of control) in PUFA-treated cells. Error bars represent SEM (n=3). *: $p<0.05$, compared to control, one-way ANOVA followed by the post-hoc Bonferroni test.

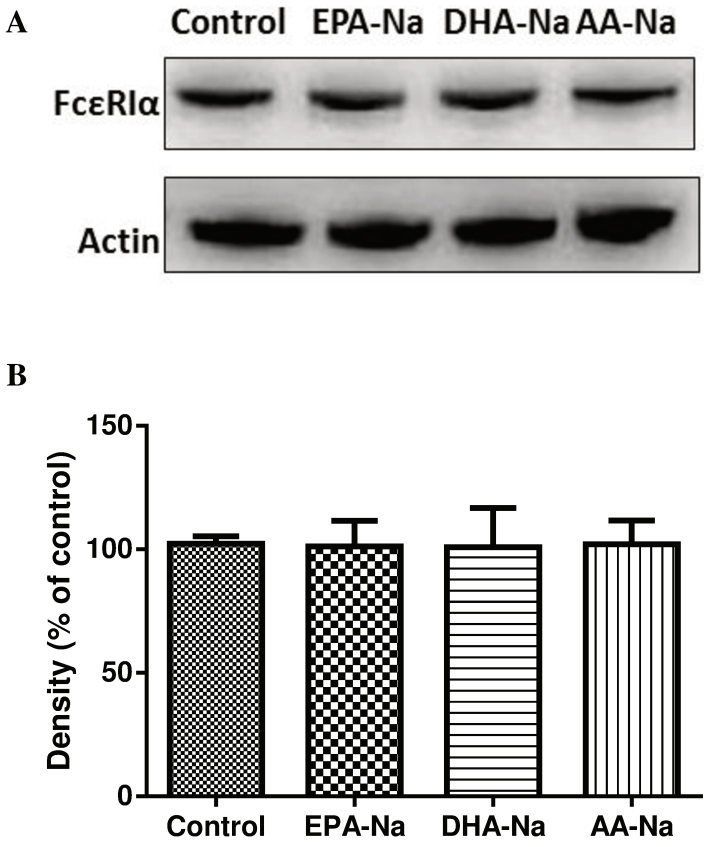

Figure 3.8 FcεRI expressions in whole cell lysates of long chain PUFA-supplemented LAD2 cells. LAD2 cells were first treated with 100 µM EPA-Na, DHA-Na, and AA-Na for 24 hr. The whole cell expression of FcεRI was measured by western blot analysis. (A) Western blot analysis of FcεRI expression in whole cell lysates of PUFA-treated LAD2 cells with actin as the loading control. Data are the representative of three independent experiments with similar results. (B) The relative density of whole cell FcεRI (% of control) in PUFA-treated cells. Error bars represent SEM (n=3).

3.4.4 Lipid raft isolation, identification and fatty acid composition determination

The raft fraction (fraction 2) expressed high level of Lyn and almost no actin (Fig. 3.9), which supports the success of lipid raft isolation.

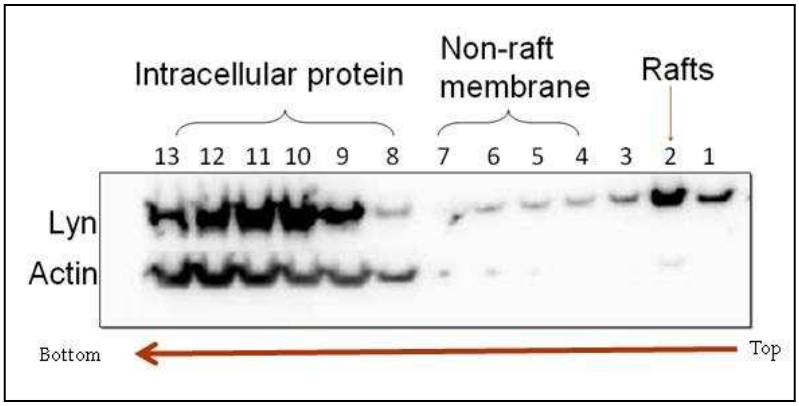

Figure 3.9 Lipid raft identification with raft marker Lyn. Lyn and actin (microfilament protein) expression in 13 fractions of sucrose gradient after centrifugation were measured by western blot analysis. Data is the representative of three independent experiments with similar results.

Eight fatty acids were identified (palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1 n-9), linoleic acid (18:2 n-6), AA (20:4 n-6), EPA (20:5 n-3), docosapentaenoic acid (DPA, 22:5 n-3), and docosahexaenoic acid (DHA, 22:6 n-3)) in lipid rafts of WT and fat-1 BMMC. The GC-MS results showed that lipid rafts of fat-1 BMMC contain significantly higher levels of EPA and DPA ($p=0.020$ and $p=0.030$), as shown in Fig. 3.10 and Table 3.1. The ratio of n-6/n-3 was significantly reduced in lipid rafts of fat-1 BMMC ($p=0.048$) compared to WT BMMC. The level of palmitic acid in lipid rafts of fat-1 BMMC was significantly deceased compared to WT BMMC ($p=0.035$).

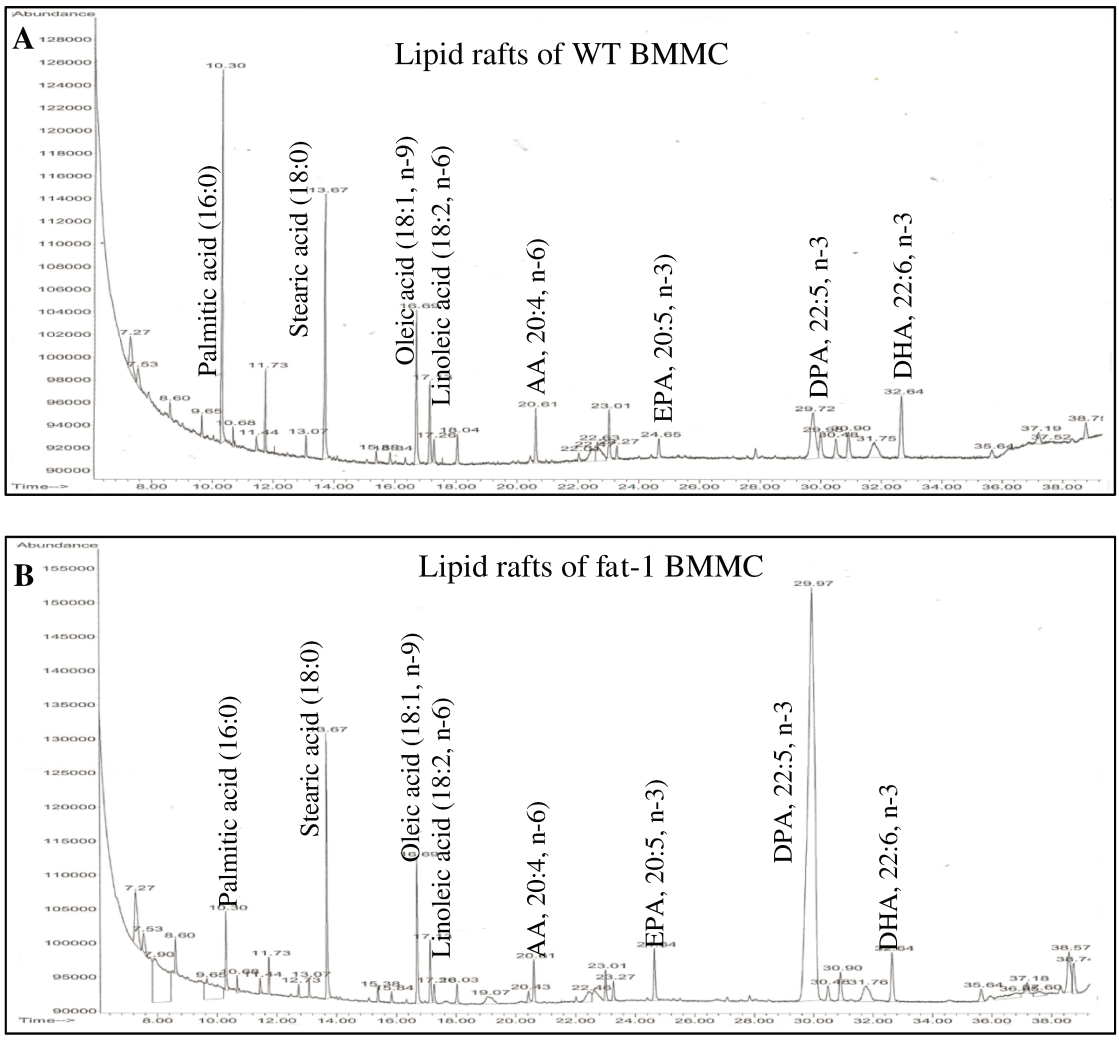

Figure 3.10 Fatty acid profiles in lipid rafts of WT and fat-1 BMMC. Eight fatty acids were identified in lipid rafts of WT (A) and fat-1(B) BMMC, which were marked besides the peaks. Data are the representative of three independent experiments. AA: Arachidonic acid; DHA: Docosahexaenoic acid; DPA: Docosapentaenoic acid; EPA: Eicosapentaenoic acid; WT: Wild type.

Table 3.1 Fatty acid composition of lipid rafts of WT and fat-1 BMMC

Fatty acids	Percentage of total fatty acids (%)	
	WT BMMC	fat-1 BMMC
16:0	23.85 ± 1.72	7.12 ± 0.99*
18:0	24.17 ± 3.15	25.74 ± 7.63
18:1 n-9	15.31 ± 2.79	16.45 ± 0.77
18:2 n-6	9.92 ± 2.84	7.12 ± 2.25
20:4 n-6 (AA)	7.23 ± 2.45	4.53 ± 1.09
20:5 n-3 (EPA)	2.12 ± 0.04	7.79 ± 0.49*
22:5 n-3 (DPA)	5.13 ± 1.76	21.01 ± 6.40*
22:6 n-3 (DHA)	12.27 ± 1.72	10.24 ± 3.81
n-6/n-3 ratio	0.87 ± 0.12	0.30 ± 0.07*

Values are mean ± SEM, n=3.

*: $p<0.05$, compared with WT BMMC, Student's t-test.

AA: Arachidonic acid; DHA: Docosahexaenoic acid; DPA: Docosapentaenoic acid; EPA: Eicosapentaenoic acid; WT: Wild type.

3.4.5 The effects of long chain n-3 PUFAs on expression of FcεRI in lipid rafts of mast cells

The expression of FcεRI in lipid rafts isolated from WT BMMC were higher (54.8 %, p=0.003) than in lipid rafts from fat-1 BMMC (Fig. 3.11). In WT BMMC, EPA and DHA supplementation decreased FcεRI expression in lipid rafts (58.2%, p=0.014; 49.1%, p=0.030, respectively; Fig. 3.12). EPA, DHA and AA supplementation also significantly reduced FcεRI expression in lipid rafts of LAD2 cells (56.3%, p=0.001; 51.7%, p<0.001; 50.2%, p<0.001; Fig. 3.13).

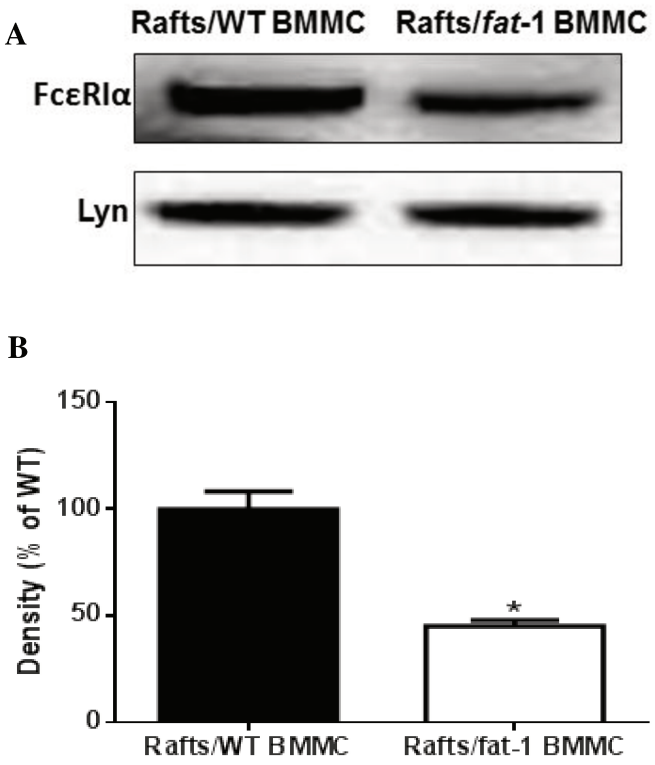

Figure 3.11 FcεRI expression in lipid rafts of WT and fat-1 BMMC. Lipid rafts were isolated with sucrose gradient centrifugation from WT and fat-1 BMMC. (A) Western blot measurement of FcεRI expression in lipid rafts of WT and fat-1 BMMC with Lyn as the loading control. Data are the representative of three independent experiments with similar results. (B) The relative density of raft FcεRI (% of wild type) in fat-1 BMMC. Error bars represent SEM (n=3). *: $p<0.05$, compared to WT BMMC (n=3), Student's t-test. WT: Wild type.

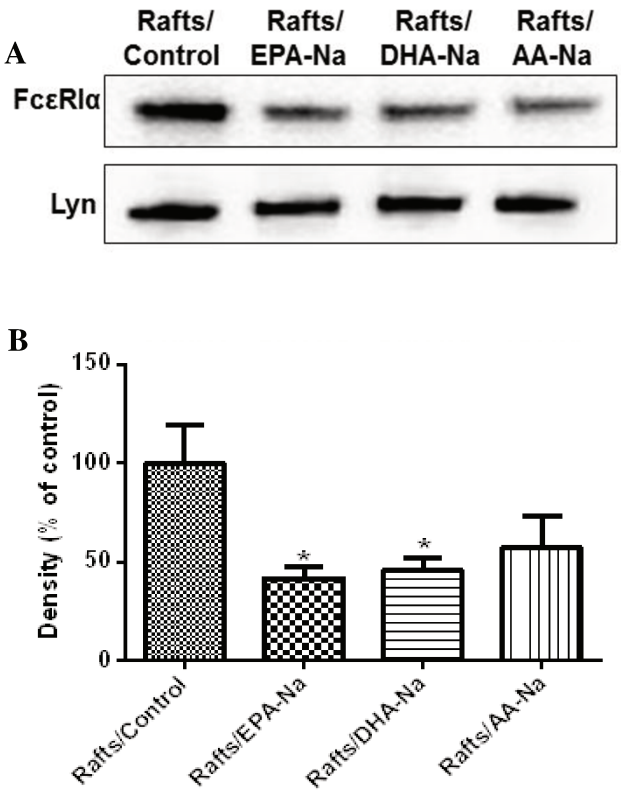

Figure 3.12 FcεRI expression in lipid rafts of long chain PUFA-supplemented WT BMMC. WT BMMC were first treated with 100 μM EPA-Na, DHA-Na, and AA-Na for 24 hr. Lipid rafts were then isolated with sucrose gradient centrifugation. (A) Western blot analysis of FcεRI expression in rafts of PUFA-supplemented BMMC with Lyn as the loading control. Data are the representative of three independent experiments with similar results. (B) The relative density of raft FcεRI (% of control) in PUFA-supplemented cells. Error bars represent SEM (n=3). *: $p<0.05$, compared with control group, one-way ANOVA followed by the post-hoc Bonferroni test.

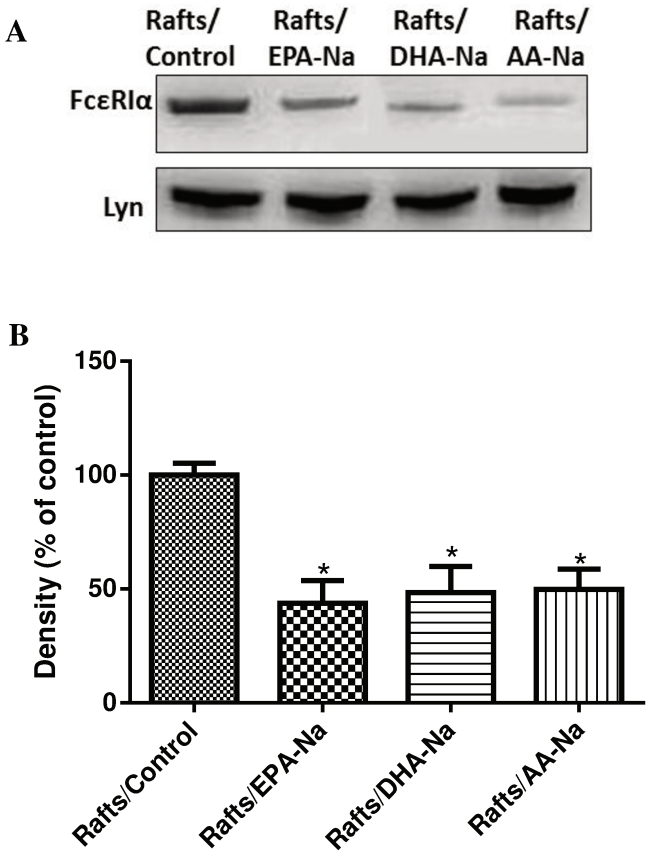

Figure 3.13 FcεRI expression in lipid rafts of long chain PUFA-supplemented LAD2 cells. LAD2 cells were first treated with 100 μM EPA-Na, DHA-Na, and AA-Na for 24 hr. Lipid rafts were then isolated with sucrose gradient centrifugation. (A) Western blot determination of FcεRI expression in rafts of PUFA-supplemented LAD2 cells with Lyn as the loading control. Data are the representative of three independent experiments with similar results. (B) The relative density of raft FcεRI (% of control) in PUFA-supplemented cells. Error bars represent SEM (n=3). *: $p<0.05$, compared with control group, one-way ANOVA followed by the post-hoc Bonferroni test.

3.4.6 The effects of long chain n-3 PUFAs on FcεRI distribution in BMMC with and without activation

In the absence of IgE/antigen, FcεRI was detected in rafts of untreated BMMC and EPA-Na-treated BMMC. However, EPA-Na treatment decreased FcεRI localization to fraction 2 ($p=0.043$). In addition, FcεRI was relocalized to fraction 4 ($p=0.026$), 5 ($p<0.001$) and 13 ($p=0.039$) in EPA-Na-treated BMMC, compared to untreated control (Fig. 3.14A and B). After IgE/antigen stimulation, in both untreated and EPA-Na-treated cells FcεRI was concentrated to fraction 2 (Fig. 3.14C and D). However, there was less FcεRI in fraction 2 ($p<0.001$) in EPA-Na-treated BMMC. In addition, FcεRI was re-localized to fraction 9 ($p=0.035$), 10 ($p=0.041$), and 13 ($p=0.044$) in EPA-Na-treated cells.

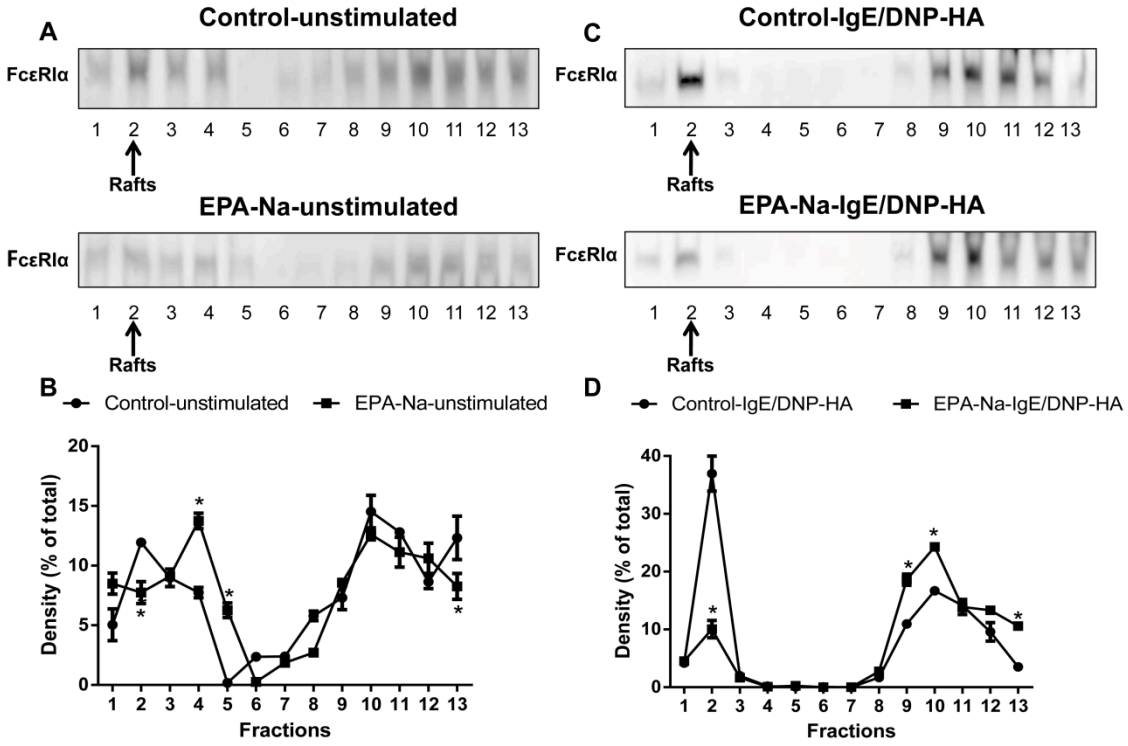

Figure 3.14 FcεRI distribution in EPA-supplemented BMMC with and without activation. (A) Western blot analysis of FcεRI distribution in 13 raft fractions of WT BMMC treated with or without 100 μM EPA-Na for 24 h. The data are the representative of three independent experiments with similar results. (B) The density percentage of FcεRI (% to total) in 13 raft fractions of WT BMMC treated with or without 100 μM EPA-Na for 24 h. (C) Western blot determination of FcεRI distribution in 13 raft fractions of WT BMMC treated with or without 100 μM EPA-Na for 24 h, followed with 500 ng/mL anti-DNP IgE sensitization for 24 h and 10 ng/mL DNP-HSA stimulation for 5 min. The data are the representative of three independent experiments with similar results. (D) The density percentage of FcεRI (% to total) in 13 raft fractions of WT BMMC treated with or without 100 μM EPA-Na for 24 h, followed with 500 ng/mL anti-DNP IgE sensitization for 24 h and 10 ng/mL DNP-HSA stimulation for 5 min. Error bars represent SEM (n=3). *: $p<0.05$, compared with control group, two-way ANOVA followed by the post-hoc Bonferroni test.

3.5 Discussion

In this study, for the first time we showed long chain n-3 PUFAs inhibit mast cell FcεRI-mediated signal transduction by reducing the expression of Lyn and LAT and modifying FcεRI receptor partitioning in lipid rafts through alteration in lipid raft property rather than by changing FcεRI expression on the cell surface and in the whole cell. This study proves that the long chain n-3 PUFA suppression of mast cell mediator release shown in chapter 2 is because of the inhibited FcεRI-mediated signal transduction.

The phosphorylation of ITAMs in β and γ chains of FcεRI by Lyn is responsible for the initiation of FcεRI-mediated signaling pathway. Lyn is thought to be phosphorylated by autophosphorylation. There are three action models for the phosphorylation of ITAMs by Lyn (Bugajev et al., 2010): 1) Receptor cross-linking allows β chain-associated Lyn to phosphorylate ITAMs of neighboring receptors. 2) FcεRI is not located close enough to Lyn to be activated in the plasma membrane. However, after receptor cross-linking, FcεRI is concentrated in rafts and becomes close enough to be phosphorylated by Lyn localized in rafts. 3) FcεRI cross-linking causes the suppression of protein tyrosine phosphatase, the negative regulator of phosphorylation.The results of this study clearly support the second model where, after IgE/antigen stimulation, FcεRI moves to the raft regions.

Phosphorylated ITAMs recruit and activate Syk, which activate some important signaling molecules, including phosphoinositide 3-kinase (PI3K), PLC-γ, SH2 domain−containing leukocyte protein 76 (SLP76), LAT, and VAV family of guanine nucleotide exchange factors (Mocsai et al., 2010). LAT also mediates the activation of

PLC-γ and another adapter protein Grb2, which induce activation of MAPK pathway (Sommers et al., 2004). The phosphorylation of Lyn, Syk and LAT were shown to be reduced in fat-1 BMMC, which is consistent with the inhibited mediator release in fat-1 BMMC (chapter 2). In the current study, fat-1 BMMC had lower Lyn and LAT whole cell expression than WT BMMC, but not Syk, which suggest that long chain n-3 PUFAs may influence FcεRI signaling in both posttranslational and transcriptional levels. Long chain n-3 PUFAs may regulate gene transcription by binding to free fatty acid receptors, GPR40 and GPR120, both are G-protein-coupled receptors (Hirasawa et al, 2008). Another way that long chain n-3 PUFAs may alter gene expression is through PPARγ, which has been confirmed to be expressed in human and murine mast cells and suggested to be involved in suppression of mast cell maturation and IgE/antigen-induced mast cell activation (Tachibana et al, 2008; Sugiyama et al, 2000).

In this study, fat-1 BMMC were shown to have an altered fatty acid profile in rafts and therefore FcεRI association with lipid rafts was disrupted. We measured the fatty acid profile in lipid rafts and showed that lipid rafts of fat-1 BMMC have a long chain n-3 PUFA-enriched fatty acid composition (i.e. increased EPA and DPA levels) compared to lipid rafts of WT BMMC. We examined FcεRI expression on the cell surface, in cell lysates, and in lipid rafts to determine the effects of long chain n-3 PUFAs on FcεRI expression. We showed that long chain n-3 PUFAs decrease FcεRI expression primarily in lipid rafts, but not on the cell surface or in whole cells in both exogenous and endogenous models. In addition, EPA-Na treatment suppressed FcεRI localization in rafts of resting cells and FcεRI shuttling to lipid rafts of activated cells, which may partially explain the reduced phosphorylation of Lyn, Syk and LAT in fat-1

BMMC. The present study is the first to show that long chain n-3 PUFAs block FcεRI association with lipid rafts in both resting and activated mast cells.

Lipid rafts are specific membrane microdomains found in plasma membranes and some intracellular membranes, such as Golgi complex, in virtually all cell types (Lingwood and Simons, 2010; Silveira et al., 2011). Currently, there are three proposed models for the involvement of lipid rafts in FcεRI-mediated signaling pathway (Draber and Draberova, 2002; Simons and Toomre, 2000). One is that receptors are located in lipid rafts or move to the raft regions where the receptor cross-linking initiates the signaling pathway (Draber and Draberova, 2002). Another model suggests that rafts move close to one another and coalesce, thus aggregating proteins including receptors (Simons and Toomre, 2000). Once the rafts are combined into a larger microdomain, they form complexes with concentrated receptors, signal adaptors, and kinases. In addition, the dephosphorylation of tyrosine kinases was suggested to be inhibited in the lipid raft local micro-environment (Barua and Goldstein, 2012). The fusion of lipid rafts can be mediated by extracellular ligands, such as antibodies, transmembrane proteins, such as LAT, and cytosolic molecules, such as Lyn, and cytoskeletal elements, such as actin (Simons and Toomre, 2000). In the first and second models, the aggregation of receptors in lipid rafts is vital for signaling initiation, which is subject to change as a result of alterations in lipid raft component, such as its fatty acid profile. The third model postulates that the initiation of signaling can be triggered by FcεRIβ chain-associated Lyn kinase and occurs outside raft regions (Draber and Draberova, 2002). Although not essential, this model concedes that the aggregation of FcεRI in lipid rafts may be important for signal amplification and maintenance under some conditions.The results of

this study showed that FcεRI can be found in raft regions of BMMC in absence of IgE/antigen. However, EPA supplementation caused FcεRI to be localized into non-raft regions. After IgE/antigen stimulation, FcεRI was concentrated to rafts and regions close to rafts in both untreated and EPA-supplemented cells. However, EPA-supplemented cells had less FcεRI moving to rafts after IgE/antigen stimulation. The data from the present study clearly supports the first model described above. However, the results of present study are not completely consistent with a previous study showing that FcεRI can only be detected in rafts after cross-linking (Field et al., 1997). However, RBL-2H3 cells, which are not a perfect analogue to mast cells, were used in that study. Different culture conditions were reported to influence FcεRI expression levels and FcεRI isoforms in RBL-2H3 cells using the same growing protocol (Froese et al., 1982). Furthermore, DNP and streptavidin caused different modes of FcεRI association with rafts in Field et al's study, which suggest FcεRI may shuttle differently to lipid rafts under different conditions.

The disruption of FcεRI localization in rafts caused by long chain n-3 PUFAs may be attributed to altered fatty acid profiles in rafts. EPA and DPA levels in rafts of fat-1 BMMC were increased and palmitic acid (16:0) level was decreased, suggesting a change in lipid raft property. In C2 mast cells, EPA and DHA were shown to increase EPA and DHA levels in raft and non-raft regions (Basiouni et al., 2012). However, the present study is the first to report that raft properties in fat-1 BMMC are altered.

In conclusion, this study demonstrates that long chain n-3 PUFAs suppress FcεRI-mediated signal transduction by inhibiting whole cell expression of Lyn and

LAT and disrupting the localization and shuttling of FcεRI to lipid rafts through alteration of lipid raft property.

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CHAPTER 4

EFFECT OF LIPID RAFT DESTABILIZATION ON ACTIVATION OF BONE-MARROW DERIVED MAST CELLS (BMMC) GENERATED FROM WILD TYPE AND fat-1 MICE

4.1 Abstract

Aim of the study: n-3 polyunsaturated fatty acids (PUFAs) inhibit mast cell activation and Fc ε receptor I (FcεRI)-mediated signal transduction by disrupting FcεRI localization and shuttling to lipid rafts. This study is to determine if lipid raft disruption influences FcεRI-mediated mediator release in wild type (WT) and long chain n-3 PUFA-enriched mast cells.

Methodology: WT and fat-1 bone marrow-derived mast cells (BMMC) were treated with methyl-β-cyclodextrin (MBCD) to deplete cholesterol. β-hexosaminidase (β-hex) release, cysteinyl leukotriene (cys-LT) synthesis, and tumor necrosis factor (TNF) and CC chemokine ligand 2 (CCL2) production were measured following activation of cells with immunoglobulin E (IgE)/2,4-dinitrophenyl-Human serum albumin (DNP-HSA). FcεRI and Lyn expression in lipid rafts following MBCD treatment was measured by western blot analysis.

Results: Following MBCD treatment, both WT and fat-1 BMMC had a very low β-hex release, cys-LT synthesis and TNF and CCL2 production. There was no significant difference on release of mediators between WT and fat-1 BMMC, except CCL2. The expressions of FcεRI and Lyn in lipid rafts were also reduced by MBCD treatment in both WT and fat-1 BMMC.

Conclusion: FcεRI-mediated mediator release is inhibited by MBCD treatment in both WT and fat-1 BMMC.

4.2 Introduction

In chapters 2 and 3, n-3 polyunsaturated fatty acids (PUFAs) were shown to inhibit mast cell degranulation, lipid-derived mediator release, and cytokine/chemokine production by suppressing Fc ε receptor I (FcεRI)-mediated signal transduction. FcεRI localization and shuttling in lipid rafts was disrupted by long chain n-3 PUFAs, but not cell surface and whole cell expression. The results suggested that lipid rafts play an important role in the inhibitory effect of long chain n-3 PUFAs on mast cell activation.

Lipid rafts are membrane domains containing high levels of cholesterol, spingolipid, glycophosphatidylinositol (GPI)-anchored proteins and acylated proteins, which have been suggested to function as platforms for signal transduction of receptors, including FcεRI (Simons and Toomre, 2000). Lipid rafts have been found in plasma membranes and Golgi complexes in almost all cell types (Nichols et al., 2001). Lipid rafts are thought to be transient, existing in a sub-second time scale (Nichols et al., 2001). Because of their special content, lipid rafts are thought to be in a liquid-ordered state featured with tightly packed and relatively slow-moving lipid (Heberle and Feigenson, 2011). This special organization also gives lipid rafts a high melting temperature and insolubility in non-ionic detergents (London and Brown, 2000).

Lipid rafts are thought to contain concentrated molecules needed for signaling, such as receptors, adaptors and kinases, so that receptor cross-linking can occur immediately after the introduction of ligands. The receptors could also be recruited to rafts after ligand encounters. In addition, the interaction between adaptors, ligands and receptors, acylated molecules, or cytoskeleton elements can cause small lipid rafts to fuse and form bigger rafts (Simons and Toomre, 2000; Staubach and Hanisch, 2011).

Lipid rafts are especially vital for FcεRI-mediated signal transduction because it requires accurate and extensive protein-protein interaction. Many membrane molecules are involved, including adapters (such as Linker for Activation of T cells (LAT)), kinases (such as Lyn), phosphatidylinositols (such as phosphatidylinositol 4,5-bisphosphate (PIP2)), and fatty acids from membrane lipid (Siraganian, 2003). Some lipid raft components have been shown to favor FcεRI-mediated signal transduction. For example, lipid rafts have high levels of Lyn expression, and the activity of Lyn in rafts is higher than in other membrane regions. The lipid raft micro-environment was suggested to inhibit Lyn dephosphorylation by phosphatase (Young et al., 2003). Another raft component, flotillin-1, was reported to colocalize with Lyn in rafts and regulate Lyn activity by direct binding to it (Kato et al., 2006).

Cholesterol is a basic structural molecule for lipid rafts. Cholesterol depletion with methyl-β-cyclodextrin (MBCD), which is a specific cholesterol binding agent, has been widely used to study lipid raft function (Hinzey et al., 2012). MBCD treatment has been shown to inhibit IgE/antigen-induced degranulation of RBL-2H3 cells (Yamashita et al., 2001). However, there is also study showing that MBCD treatment has no effect on IgE/antigen-induced mast cell degranulation, but inhibit FcεRI phosphorylation by blocking the interplay between FcεRI and Lyn (Sheets et al., 1999).

My previous study has shown that long chain n-3 PUFAs inhibit mast cell mediator release induced by FcεRI-activation and suppress FcεRI-mediated signal transduction, partly by disrupting FcεRI localization and shuttling to lipid rafts (chapter 2 and 3). Thus, we hypothesized that the MBCD pretreatment inhibits FcεRI-mediated mediator release in both normal mast cells and long chain n-3 PUFA-enriched mast cells. WT

and fat-1 bone marrow-derived mast cells (BMMC) activation was evaluated after MBCD treatment.

4.3 Materials and Methods

4.3.1 Growth of WT and fat-1 BMMC

The method was the same as described in chapter 2, section 2.3.2.

4.3.2 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay

XTT assay was performed in BMMC after 10 mM MBCD (Sigma-Aldrich, Oakville, ON) treatment for 30 minutes (min) with the method described in chapter 2, section 2.3.6.

4.3.3 Degranulation assay

BMMC were sensitized with anti-2,4-dinitrophenyl (DNP) immunoglobulin E (IgE) (Sigma-Aldrich) for 24 hours (hr), followed by treatment with 10 mM MBCD (Sigma-Aldrich) for 30 min. Cells were then activated with 0.001, 0.01, 0.1, 1, 10, 100, 200 or 500 ng/mL of DNP-human serum albumin (HSA) for 30 min (Sigma-Aldrich). Degranulation was determined by measuring β-hexosaminidase (β-hex) release with the method described in chapter 2, section 2.3.9.

4.3.4 Enzyme-linked immunosorbent assay (ELISA) for cysteinyl leukotrienes (cys-LTs), tumor necrosis factor (TNF) and CC chemokine ligand 2 (CCL2)

BMMC were sensitized with anti-DNP IgE (Sigma-Aldrich) for 24 hr, followed by treatment with 10 mM MBCD (Sigma-Aldrich) for 30 min. Cells were then activated by 1, 10, 100, 200 or 500 ng/mL of DNP-HSA (Sigma-Aldrich) for 3 hr for LT release

and 6 hr for cytokine/chemokine production. The release of cys-LT, TNF, CCL2 were measured by ELISA with the method described in chapter 2, section 2.3.10.

4.3.5 Lipid raft isolation and western blot analysis

Lipid rafts were isolated from BMMC following treatment with 10 mM MBCD (Sigma-Aldrich) for 30 min with the method described in chapter 3, section 3.3.6. Western blot analysis was performed with the method described in chapter 3, section 3.3.8.

4.3.6 Statistical analysis

The differences on cell viability before and after MBCD treatment were determined using Student's t-test. Two-way analysis of variance (ANOVA) followed by the post-hoc Bonferroni test were used to evaluate the difference in mediator release between WT and fat-1 BMMC with and without MBCD treatment. Statistical significance was set at $p<0.05$. Statistical analysis was performed in SPSS 11.5 statistical software package (IBM Corporation, Armonk, NY).

4.4 Results

4.4.1 The effect of MBCD treatment on BMMC viability

In both WT and fat-1 BMMC, there was no significant difference in viability between untreated cells and cells treated with 10 mM MBCD for 30 min (Fig. 4.1).

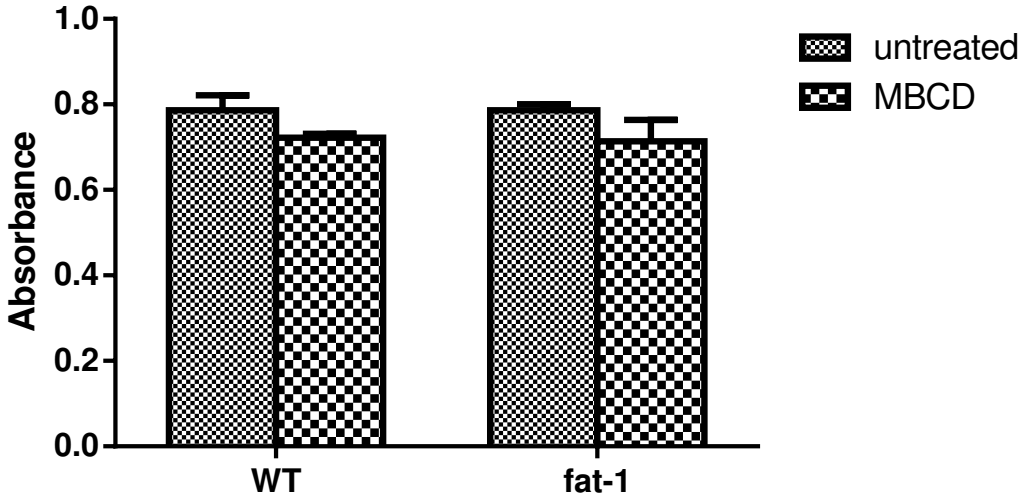

Figure 4.1 Cell viability of BMMC after MBCD treatment. WT and fat-1 BMMC viability were evaluated by XTT assay after 30 min 10 mM MBCD treatment and is compared to untreated control. Error bars represent standard error of the mean (SEM) (n=3). XTT: 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; WT: Wild type.

4.4.2 The effect of MBCD treatment on FcεRI-mediated BMMC degranulation

Following MBCD treatment, the β-hex release level (% of total) was low (<20%, maximally) in both types of BMMC (Fig. 4.2A). Control WT and fat-1 BMMC that received no MBCD treatment had a maximum of 41.6% and 33.3% β-hex release respectively (Fig. 4.2B and C). In addition, there was no significant difference in β-hex release between MBCD-treated WT and fat-1 BMMC in different concentrations of DNP-HSA (Fig. 4.2A). Without the activation by DNP-HSA, MBCD treatment alone caused significantly higher β-hex release than untreated cells in both WT and fat-1 BMMC ($p=0.036$ and $p=0.041$) (Fig. 4.2B and C). In response to 10, 100, 200, and 500 ng/mL DNP-HSA stimulation, both MBCD-treated WT and fat-1 BMMC produced significantly less β-hex compared to untreated controls ($p<0.05$) (Fig. 4.2B and C).

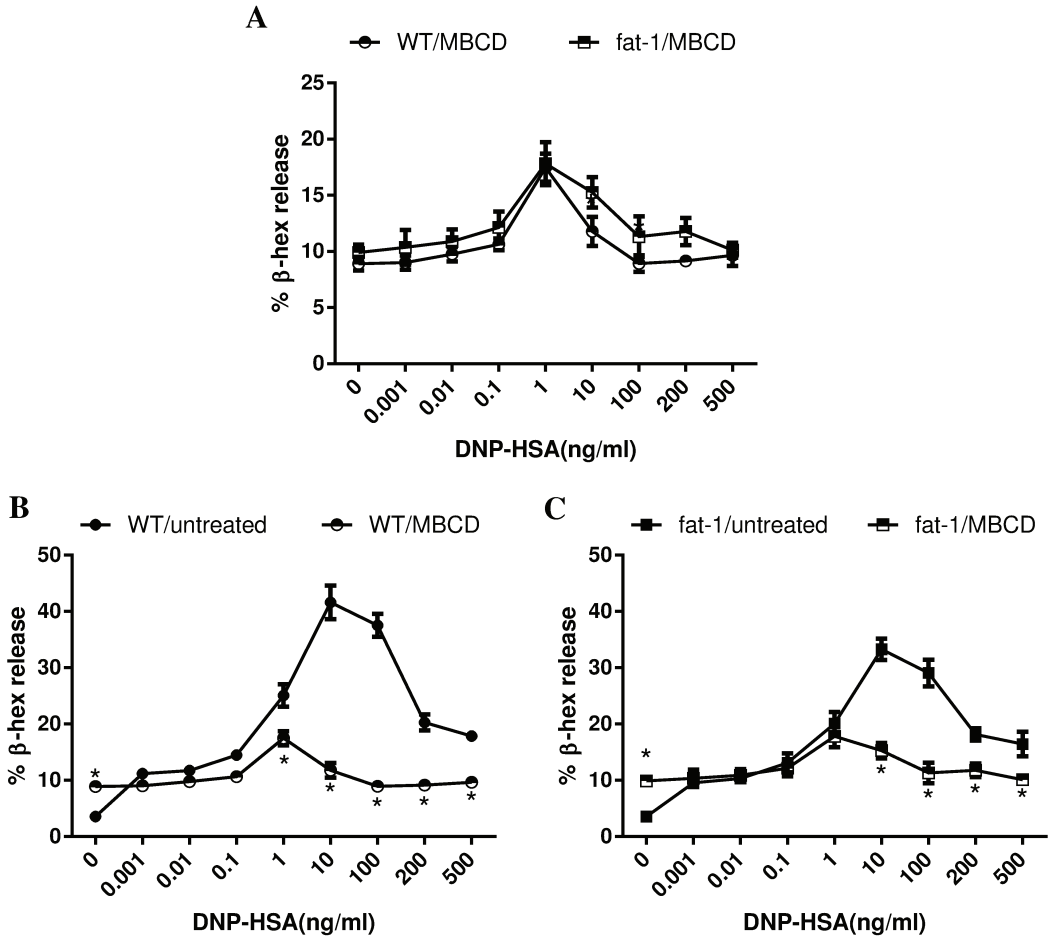

Figure 4.2 FcεRI-mediated degranulation of BMMC after MBCD treatment. WT and fat-1 BMMC were sensitized with 500 ng/mL anti-DNP IgE for 24 hr. After 30 min MBCD treatment, cells were stimulated with indicated concentrations of DNP-HSA for 30 min. (A) β-hex release of WT and fat-1 BMMC after MBCD treatment. (B) β-hex release of WT BMMC with and without MBCD treatment. (C) β-hex release of fat-1 BMMC with and without MBCD treatment. Error bars represent SEM (n=3). *: $p<0.05$, compared to WT BMMC (A) or untreated control (B, C), two-way ANOVA followed by the post-hoc Bonferroni test. WT: Wild type.

4.4.3 The effect of MBCD treatment on FcεRI-mediated cys-LT release by BMMC

Similar to the degranulation results, the cys-LTs release level was very low (<70 pg/mL, maximally) in IgE/DNP-HSA-stimulated WT and fat-1 BMMC after MBCD treatment (Fig. 4.3A). Without MBCD treatment, WT and fat-1 BMMC produced maximally 1612.6 and 1181.3 pg/mL cys-LTs respectively (Fig. 4.3B and C). There was no significant difference between WT and fat-1 BMMC in IgE/DNP-HSA-induced cys-LTs release after MBCD treatment (Fig. 4.3A). In response to 1, 10, 100, 200, and 500 ng/mL DNP-HSA stimulation, both MBCD-treated WT and fat-1 BMMC produced significantly less cys-LTs compared to their untreated control ($p<0.05$) (Fig. 4.3B and C).

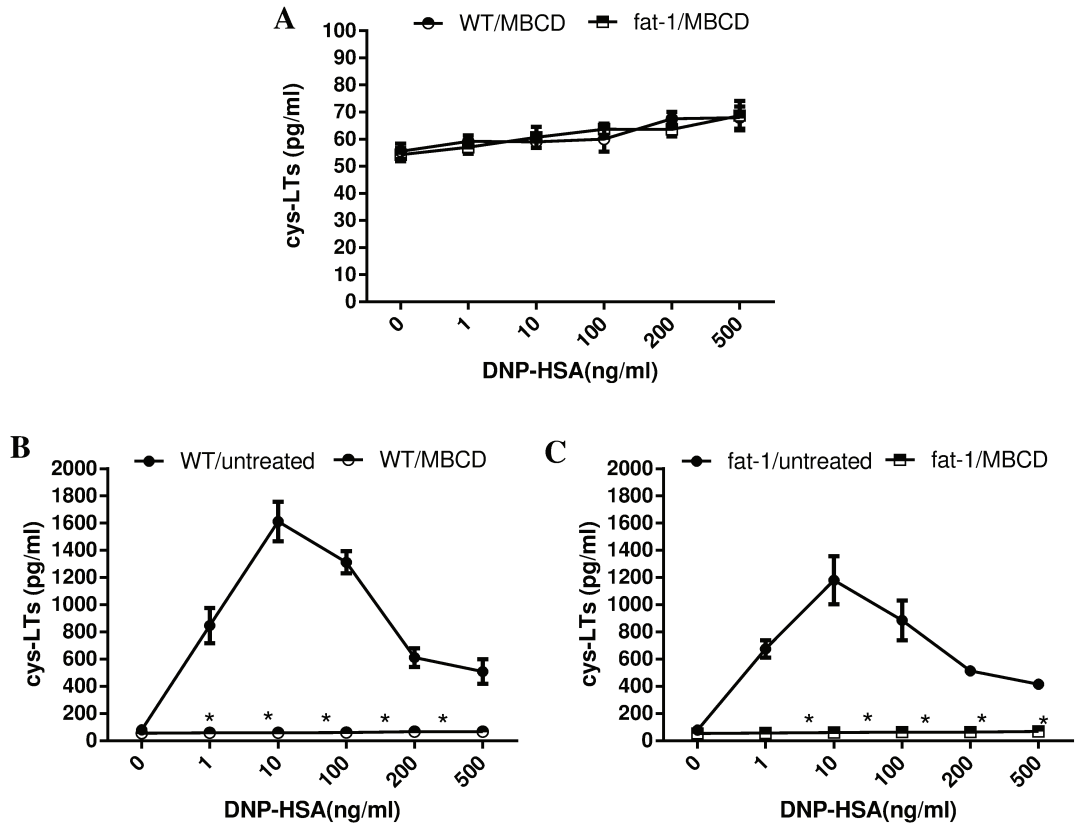

Figure 4.3 FcεRI-mediated cys-LT release by BMMC after MBCD treatment. WT and fat-1 BMMC were sensitized with 500 ng/mL anti-DNP IgE for 24 hr. After 30 min MBCD treatment, cells were stimulated with indicated concentrations of DNP-HSA for 3 hr. (A) ELISA measurement of cys-LT release by WT and fat-1 BMMC after MBCD treatment. (B) ELISA determination of cys-LT release by WT BMMC with and without MBCD treatment. (C) ELISA measurement of cys-LT release by fat-1 BMMC with and without MBCD treatment. Error bars represent SEM (n=3). *: $p<0.05$, compared to WT BMMC (A) or untreated control (B, C), two-way ANOVA followed by the post-hoc Bonferroni test. WT: Wild type.

4.4.4 The effect of MBCD treatment on FcεRI-mediated cytokine/chemokine production by BMMC

IgE/DNP-HSA-induced TNF production by WT and fat-1 BMMC was very low (<15 pg/mL, maximally) following MBCD treatment, and no significance was found between WT and fat-1 BMMC in TNF production after MBCD treatment (Fig. 4.4A). Without MBCD treatment, WT and fat-1 BMMC produced a maximum of 982.8 and 738.0 pg/mL TNF respectively (Fig. 4.4B and C). In response to 1, 10, 100, 200, and 500 ng/mL DNP-HSA stimulation, both MBCD-treated WT and fat-1 BMMC produced significantly less TNF compared to their untreated control ($p<0.005$) (Fig. 4.4B and C).

Following MBCD treatment, the CCL2 production in response to IgE/DNP-HSA was less than 100 pg/mL at maximum, in both WT and fat-1 BMMC (Fig. 4.5A). Without MBCD treatment, WT and fat-1 BMMC produced maximally 1640.3 and 1131.3 pg/mL CCl2 respectively (Fig. 4.5B and C). MBCD-treated fat-1 BMMC produced less CCl2 (51.5 pg/mL compared to 85.9 pg/mL, $p=0.001$) in response to 10 ng/mL DNP-HSA compared to MBCD-treated WT BMMC (Fig. 4.5A). In response to 1, 10, 100, 200, and 500 ng/mL DNP-HSA stimulation, both MBCD-treated WT and fat-1 BMMC produced significantly less CCL2 compared to their untreated controls ($p<0.005$) (Fig. 4.5B and C).

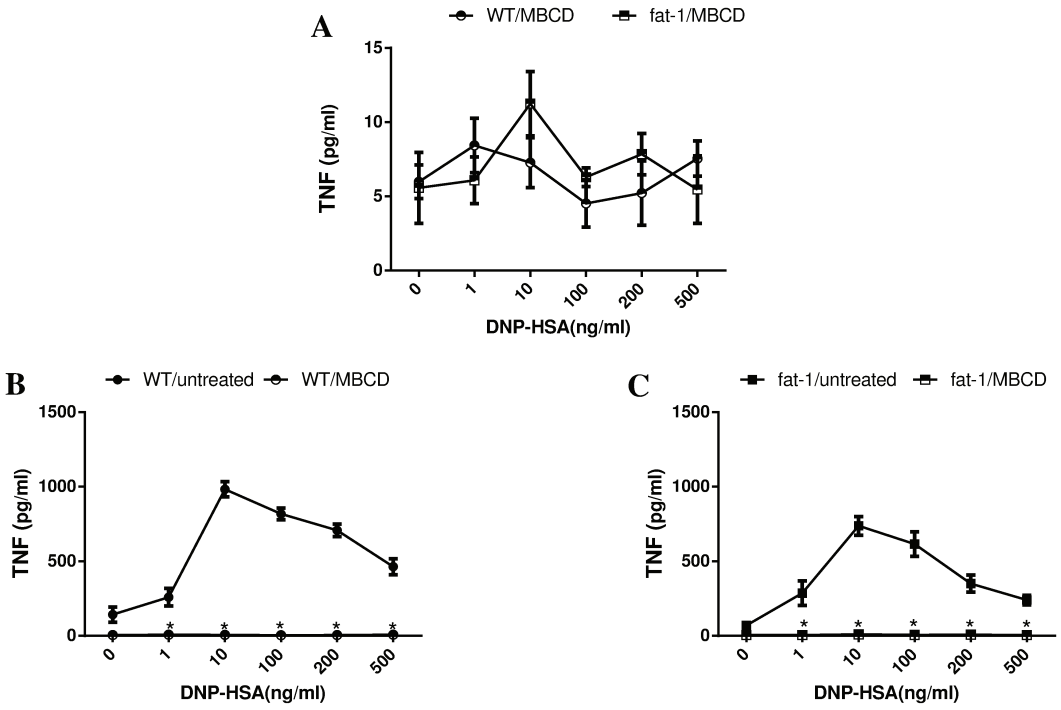

Figure 4.4 FcεRI-mediated TNF production by BMMC after MBCD treatment. WT and fat-1 BMMC were sensitized with 500 ng/mL anti-DNP IgE for 24 hr. After 30 min MBCD treatment, cells were stimulated with indicated concentrations of DNP-HSA for 6 hr. (A) ELISA measurement of TNF production by WT and fat-1 BMMC after MBCD treatment. (B) ELISA determination of TNF production by WT BMMC with and without MBCD treatment. (C) ELISA measurement of TNF production by fat-1 BMMC with and without MBCD treatment. Error bars represent SEM (n=3). *: $p<0.005$, compared to WT BMMC (A) or untreated control (B, C), two-way ANOVA followed by the post-hoc Bonferroni test. WT: Wild type.

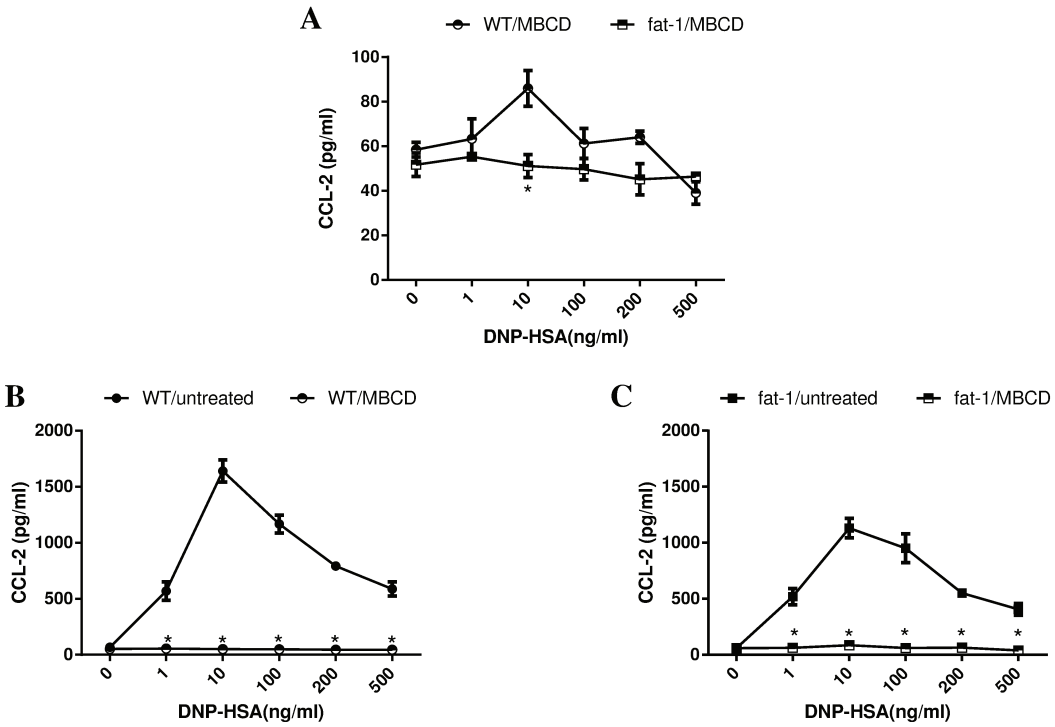

Figure 4.5 FcεRI-mediated CCL2 release by BMMC after MBCD treatment. WT and fat-1 BMMC were sensitized with 500 ng/mL anti-DNP IgE for 24 hr. After 30 min MBCD treatment, cells were stimulated with indicated concentrations of DNP-HSA for 6 hr. (A) ELISA measurement of CCL2 production by WT and fat-1 BMMC after MBCD treatment. (B) ELISA determination of CCL2 production by WT BMMC with and without MBCD treatment. (C) ELISA measurement of CCL2 production by fat-1 BMMC with and without MBCD treatment. Error bars represent SEM (n=3). *: $p<0.005$, compared to WT BMMC (A) or untreated control (B, C), two-way ANOVA followed by the post-hoc Bonferroni test. WT: Wild type.

4.4.5 The effect of MBCD treatment on FcεRI and Lyn localization in lipid rafts of BMMC

Without MBCD treatment, the expression of FcεRI in lipid rafts of WT BMMC was higher (54.8 %, p=0.003) than in lipid rafts of fat-1 BMMC (Fig. 4.6A and B left). However, after MBCD treatment, the expression of FcεRI and Lyn were dramatically decreased in lipid rafts of WT and fat-1 BMMC compared to their untreated control (Fig. 4.6A). In addition, no significant difference in raft FcεRI expression between MBCD-treated WT and fat-1 BMMC was found (Fig. 4.6B right).

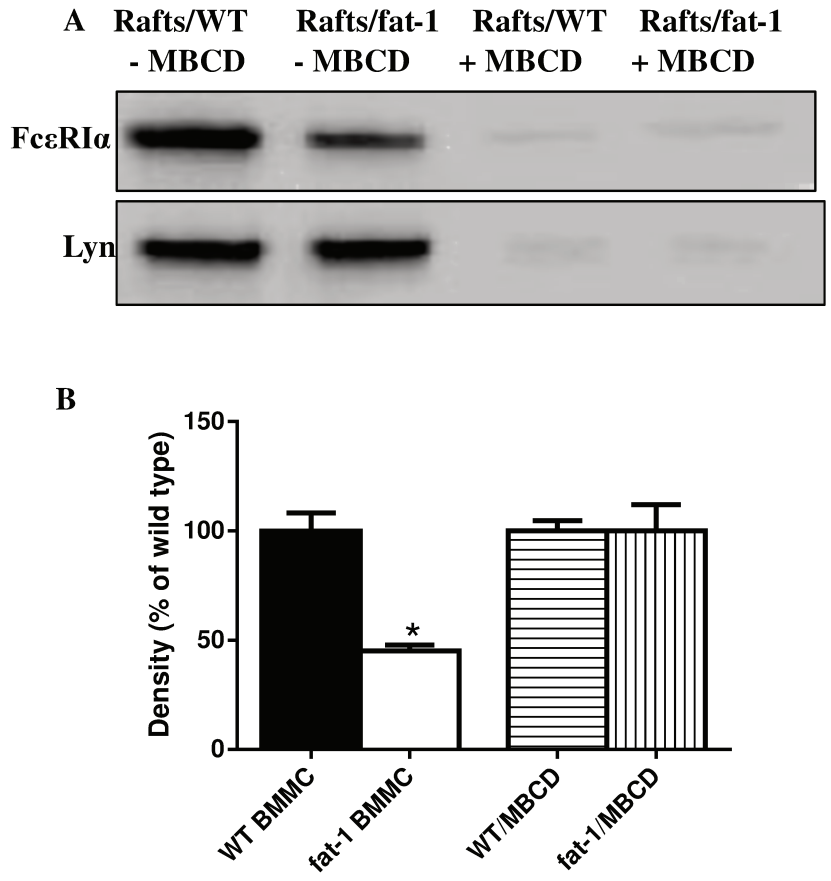

Figure 4.6 FcεRI and Lyn expression in rafts of BMMC after MBCD treatment. Lipid rafts were isolated using sucrose gradient centrifugation from WT and fat-1 BMMC with or without 30 min MBCD treatment. (A) Western blot analysis of FcεRI and Lyn expressions in rafts of WT and fat-1 BMMC with or without MBCD treatment. Data are the representative of three independent experiments with similar results. (B) The relative density (% of wild type) of raft FcεRI in fat-1 BMMC with and without MBCD treatment. Error bars represent SEM (n=3). *: $p<0.05$, compared to WT control, Student's t-test. WT: Wild type.

4.5 Discussion

In this study, both WT and fat-1 BMMC had an inhibited response to IgE/antigen following MBCD treatment. IgE/antigen-induced degranulation, lipid-derived mediator release and cytokine/chemokine production were all disrupted by MBCD pretreatment due to reduced FcεRI and Lyn localization in rafts. The difference in mediator release and FcεRI raft expression between WT and fat-1 BMMC detected in chapters 2 and 3 disappeared after MBCD treatment, suggesting that cholesterol has a greater influence than long chain n-3 PUFAs on FcεRI localization in lipid rafts and FcεRI-mediated mediator release of mast cells.

Lipid rafts are involved in the processes of endocytosis, exocytosis, receptor signaling, and pathogen entry and replication (Calder and Yaqoob, 2007; Hanzal-Bayer and Hancock, 2007; Zajchowski and Robbins, 2002). In mast cells, lipid rafts are required for FcεRI endocytosis, ubiquitination and degradation in lysosomes (Silveira et al., 2011). A lipid raft component, ganglioside derived from GD1b, is expressed by mast cells in all stages of maturation (Zuberbier et al., 1999), and is internalized together with FcεRI during receptor endocytosis (Mazucato et al., 2011). Lipid rafts are also associated with soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNAREs) which aggregates proteins required for exocytosis in mast cells (Puri and Roche, 2006). However, lipid rafts are especially important for FcεRI-mediated signal transduction, which occurs at a sub-second scale, and contains collections of secondary messengers required for signaling initiation and propagation (Siraganian, 2003). FcεRI was found to co-localize with Lyn in lipid rafts (Wilson et al., 2000) which is anchored to the inner leaflet of the plasma membrane through myristate and palmitate chains

(Kovarova et al., 2001). Within the lipid raft, a direct interaction between Lyn and the FcεRI β chain may exist (Vonakis et al., 1997). Interestingly, the activity of Lyn in lipid rafts is much higher than outside of lipid rafts (Young et al., 2003), which suggests that lipid rafts may not be essential for FcεRI signal transduction but necessary for an optimal response.

Cholesterol is an essential raft component required for raft stabilization (Lingwood and Simons, 2010). Exogenous cholesterol was shown to activate CPII mouse mast cells by inducing activation of P38 and FosB following incorporation to rafts (Baumruker et al., 2003). As small polar molecules that possess hydrophobic pockets to capture cholesterol, β-cyclodextrins can remove cholesterol in cells. Cholesterol depletion caused by β-cyclodextrins can be reversed by exogenous cholesterol treatment (Barman and Nayak, 2007). MBCD is relatively less-toxic among β-cyclodextrins (Kiss et al., 2007), which was widely used to explore the role of lipid rafts in functions of various types of cells. It was reported that MBCD treatment disrupted PIP2 localization in rafts, suppressed RBL-2H3 cell degranulation and calcium influx induced by IgE/antigen (Yamashita et al., 2001). However, the phosphorylation of FcεRI subunits and phospholipase C-γ (PLC-γ) were not influenced (Yamashita et al., 2001). There were conflicting results from another study using the same type of cell, which showed that MBCD treatment does not significantly inhibit IgE/antigen induced degranulation, but instead enhances calcium ionophore-induced degranulation (Sheets et al., 1999). The phosphorylation of FcεRI was inhibited by MBCD treatment, and the interaction of FcεRI, Lyn and rafts was disrupted (Sheets et al., 1999). RBL-2H3 cells were reported to express different level of FcεRI in different culture conditions (Froese et al., 1982),

which may result in the difference between results described above. In 3β-hydroxysterol Δ7-reductase (DHCR) deficient mast cells, whose membranes contain depleted cholesterol and accumulated cholesterol precursor 7-dehydrocholesterol (DHC), lipid raft stability was disrupted and the expression of Lyn in rafts was suppressed (Kovarova et al., 2006). However, hyper-degranulation was observed as a result of decreased phosphorylation of Csk-binding protein, which is dependent on Lyn and function as a negative regulator for degranulation-promoting Fyn activity (Kovarova et al., 2006).

The current study showed that MBCD treatment inhibited leukotriene production in both WT and fat-1 BMMC. A similar effect was observed in RBL-2H3 cells (You et al., 2007). After MBCD incubation, the leukotriene synthesis induced by calcium ionophore was diminished, which can increase the intracellular calcium level by facilitating the cross of calcium to cell membrane and entry to cells. This observation was reversed by cholesterol treatment (You et al., 2007). A critical molecule involved in leukotriene synthesis, 5-lipoxygenase activating protein (FLAP), was found to co-localize with a lipid raft marker, flotillin-1, suggesting the localization of FLAP in lipid rafts (You et al., 2007). Thus, cholesterol depletion may directly down-regulate leukotriene production by disrupting FLAP function in rafts.

The results of this study also demonstrated reduced cytokine/chemokine production in MBCD-treated mast cells, which was not reported in any other previous studies on mast cells. However, similar effects can be found in macrophages. MBCD treatment was seen to decrease TNF expression in both mRNA and protein levels in RAW264.7 murine macrophage cells stimulated with lipopolysaccharide (LPS) (Nishiyama et al., 2008).The result of this study also showed that the CCL2 production

after MBCD treatment is lower in fat-1 BMMC than WT, suggesting cholesterol and long chain n-3 PUFA together may result in more impaired mast cell activation than cholesterol alone. This study is the first report to show an inhibitory effect of MBCD treatment in FcεRI-mediated activation of primary WT and long chain n-3 PUFA-enriched mast cells. In Kovarova's study (Kovarova et al., 2006), the mice were genetically modified to have depleted cholesterol together with accumulated 7-DHC, which might have an influence on cell function.

FcεRI and Lyn expression in lipid rafts was disrupted by MBCD treatment in both WT and fat-1 BMMC, which might be responsible for the results shown in this study. Disruption of receptor expression in lipid rafts by cholesterol depletion is a common phenomenon (Simons and Toomre, 2000; Zidovetzki and Levitan, 2007). For instance, cholesterol is not only connected to the production of leukotrienes, but also involved in a cell's response to LTs. LTB_4 receptor BLT-1 is reported to be expressed in lipid rafts together with flotillin-1 in human neutrophils (Sitrin et al., 2006). MBCD treatment disrupted BLT-1 raft expression and suppressed neutrophil activation induced by LTB_4 (Sitrin et al., 2006). However, the current study is the first one to report disrupted FcεRI expression in rafts of the primary mast cells by MBCD treatment. The result of this study also showed that FcεRI and Lyn expression in lipid rafts was equally reduced in WT and fat-1 BMMC by MBCD treatment, even though the fat-1 BMMC contain less FcεRI in lipid rafts than WT (Fig. 3.11), suggesting MBCD-caused defect on cholesterol may have stronger effect on protein localization in lipid rafts than long chain n-3 PUFA incorporation.

In summary, these results showed that MBCD pretreatment suppresses IgE/antigen-induced mast mediator release, and disrupt raft localization of FcεRI and Lyn in both WT and fat-1 BMMC, suggesting a greater influence of cholesterol on lipid raft function than long chain n-3 PUFA. Both WT and long chain n-3 PUFA-enriched mast cells require cholesterol for proper response to IgE/antigen. The importance of lipid rafts in FcεRI-mediated mast cell mediator release is supported by this study.

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CHAPTER 5

GENERAL DISCUSSION, CONCLUSIONS, AND FUTURE DIRECTIONS

5.1 General discussion

5.1.1 Lipid raft functions in immune responses

As introduced in chapter 1, lipid rafts have been proposed to take part in innate and adaptive immune responses by sequestering lipids and proteins in macrophages, T cells and B cells (Simons and Toomre, 2000). This study supports the role of lipid rafts in the regulation of signal transduction in mast cells. Fc ε receptor I (FcεRI) can be found in the lipid rafts of resting mast cells and concentrates in rafts after cell activation. This supports the first and second model of lipid rafts in signal transduction, described in the chapter 1 (section 1.3.2), emphasizing the presence and quick partitioning of receptors to lipid rafts. Lipid rafts, with their concentrated FcεRI and Lyn, are important for FcεRI-mediated signal transduction which is extremely quick (Liu et al., 2013). The preformed mediators in granules of mast cells can be released within seconds after encountering an antigen. In the current study, long chain n-3 PUFAs were shown to inhibit immunoglobulin E (IgE)/antigen-induced release of mast cell mediators and FcεRI-mediated signal transduction by disrupting FcεRI localization in rafts of resting cells and FcεRI shuttling to rafts in activated cells, and expression of Lyn and linker of activated T cells (LAT) (chapter 2 and 3).

5.1.2 Lipid raft functions in mast cells

This study supports the important function of lipid rafts in FcεRI-mediated signal transduction and activation of mast cells. However, the results of the present study are not entirely consistent with some previous studies.The results of this study suggest that FcεRI can be found in lipid rafts in resting mast cells, which was not seen in a previous

study in which FcεRI was detected in lipid rafts of RBL-2H3 basophilic leukemia cells only after cross-linking (Field et al., 1997). RBL-2H3 cells, which are generated from basophilic leukemia, are not perfect analogies to mast cells (Passante et al., 2009). For example, RBL-2H3 cells do not respond well to lipopolysaccharide (LPS) because of the absence of cluster of differentiation (CD) 14 expression (Passante et al., 2009), which is present in both human and mouse mast cells (McCurdy et al., 2001; Welker et al., 2000). Although RBL-2H3 cells share some characteristics with mucosal mast cells and are easy to culture, conflicting results using this cell line are commonly seen in different laboratories, possibly because they are sensitive to different culture conditions (Froese et al., 1982). For instance, FcεRI expression in RBL-2H3 cells from four different laboratories cultured with the same protocol were reported to express different levels of FcεRI with inconsistent molecular weights (Froese et al., 1982).

Another explanation for the difference between the results of the present study and Field's study is that the lipid raft isolation method is different. I modified a lipid raft isolation protocol published by Dr. D.A. Brown in 2002 (Brown, 2002), which has been used by many subsequent studies with or without minor modifications (Lopez et al., 2012). Field et al (Field et al., 1997) used 0.05% Triton X-100 to lyse the cells, and the sucrose gradient was set up as 80%, 60%, 40% , 30%, 20%, and 10%. While I used 1% Triton X-100 with two additional protease inhibitors, leupeptin and pepstatin, to lyse the cells. Leupeptin and pepstatin may provide further protection for raft proteins from degradation, which is vital in the present study because mast cells are known to contain many types of proteases (Gilfillan and Tkaczyk, 2006). The sucrose gradient in the present study was set at 44%, 35% and 5%. Furthermore, SW41 rotor was used in

the present study for ultracentrifuge at 100,000 × g for 3 hours (hr), while Field et al used a SW60.1 rotor at 250,000 × g for 12-18 hr. In the present study, 30 minutes (min) methyl-β-cyclodextrin (MBCD) treatment inhibited IgE/antigen-induced degranulation, lipid-derived mediator release and cytokine/chemokine production in both WT and fat-1 bone-marrow-derived mast cells (BMMC), confirming that cholesterol, an important lipid raft component, is essential for FcεRI-mediated mediator release of mast cells.

5.1.3 Fatty acids/lipid effects on mast cells

The present study is the first to provide comprehensive evidence that n-3 polyunsaturated fatty acids (PUFAs) have an effect on mast cell activation. In addition, FcεRI association with rafts, but not expression of FcεRI on cell surface and whole cell lysates, is disrupted by n-3 PUFA incorporation, thus changing the property of the rafts. In addition, endogenously produced long chain n-3 PUFAs actually decreased Lyn and LAT whole cell expression in mast cells, which may be the result of binding of long chain n-3 PUFAs on free fatty acid receptors and peroxisome proliferator-activated receptors (Calder, 2013).

Lipids other than n-3 PUFAs may also have modulating roles in mast cell function. In C2 cells, 8-day treatment of linoleic acid (18:2 n-6) enhanced tryptase activity in resting cells and amplified histamine release in response to mastoparan (Gueck et al., 2004), a peptide toxin from wasp venom. Phospholipase D (PLD) is an enzyme that catalyzes the hydrolysis of a major membrane phospholipid, phosphatidylcholine, to phosphatidic acid and choline. Phospholipase D is believed to be an important player in exocytosis and vesicle trafficking (Shen et al., 2001). Arachidonic acid (AA, n-6) was

reported to protect PLD activity in the presence of PLD inhibitors in C2 canine mastocytoma cells (Basiouni et al., 2013). AA treatment was also reported to increase AA levels in both raft and non-raft membrane regions of C2 mast cells (Basiouni et al., 2012). After a 24 hr AA incubation, >50% of AA was shown to incorporate into phosphatidylcholine in membranes of human lung mast cells, and IgE/anti-IgE-induced histamine release was significantly higher than control (Peters et al., 1984).

Another lipid type that has gained some interest in this field is cholesterol, as described in chapter 4. The results showed that MBCD treatment inhibited IgE/antigen-induced mediator release in both WT and fat-1 BMMC. Similar to the results of the present study, cholesterol depletion was shown to inhibit mast cell activation by suppressing FcεRI phosphorylation and Lyn localization in rafts (Kovarova et al., 2006). In addition, exogenous cholesterol treatment lead to an increased cholesterol level in rafts, and induced degranulation and leukotriene production from CPII mouse mast cells by causing P38 kinase activation (Baumruker et al., 2003).

5.1.4 Clinical meaning of this study for patients with mast cell-mediated disease

This study showed that long chain n-3 PUFAs can modulate lipid raft function and inhibit mast cell activation mediated by FcεRI, which is the principle activating pathway of mast cells. In addition, lipid rafts are involved in a broad range of signaling pathways as described in chapter 1, section 1.3.2. Therefore, long chain n-3 PUFAs might be a valuable supplement for mast cell-mediated diseases, such as allergic asthma, urticaria, mastocytosis and atopic dermatitis.

N-3 PUFAs may also have a potential beneficial effect on cancer. In recent years, inflammation has started to be considered as a fundamental and universal event in cancer development (Grivennikov et al., 2010). Furthermore, the role of mast cells in cancer development is becoming recognized (Khazaie et al., 2011). Mast cells are found to accumulate around tumor sites in both human and rodent cancers, including breast, lung, liver and prostate cancer (Nonomura et al., 2007; Ranieri et al., 2009; Stoyanov et al., 2012; Terada and Matsunaga, 2000). The roles of mast cells in cancer are thought to be multifaceted. An increased number of mast cells in peritumoral tissue in prostate cancer is associated with a bad prognosis, whereas increased numbers of mast cells in intratumoral tissue is associated with a good prognosis (Fleischmann et al., 2009; Johansson et al., 2010). There was also a study showing that mast cell numbers in intratumoral border zone are correlated with a bad prognosis of pancreatic ductal adenocarcinoma (Cai et al., 2011). However, mast cell numbers in the intratumoral center zone and the peritumoral zones were not associated with the prognosis of pancreatic cancer (Cai et al., 2011), suggesting that the role of mast cell in cancer might be dependent on the specific cancer types. The consumption of long chain n-3 PUFAs has been associated with lower risk of breast cancer (Zheng et al., 2013). Because long chain n-3 PUFAs are food components with large applicable safe-dose ranges, they have some overwhelming merits to be used in disease treatment. First, they can be used for long term with almost no or negligible side effects, making them suitable for chronic conditions. Second, they may help reduce the use of medicines with well-documented undesirable side effects. For example, inhaled and oral corticosteroids are very effective long-term control medications for asthma, but the side effects of

corticosteroids can include growth inhibition, adrenal suppression, dermal thinning, hypertension, cataracts, and muscle weakness (Dahl, 2006). Chronic use of corticosteroids may also lead to immunologic attenuation (Walker and Edwards, 2013). Reduction in the corticosteroid dosage can contribute to an improvement in the quality of life in patients.

5.1.5 n-3 PUFAs and lipid rafts in cells other than mast cells

The importance of lipid rafts in modulating receptor signal transduction was observed in many types of cells besides mast cells, suggesting that their function is ubiquitously present in a broad range of cells (Simons and Toomre, 2000). Docosahexaenoic acid (DHA) supplementation was reported to increase DHA levels in lipid rafts of T cells, thus decreasing IL-2 receptor and STAT5a/b expression in rafts (Li et al., 2005). In colonic epithelial cells, DHA was shown to change lipid raft composition and suppress activation of ERK1/2, STAT3, and mammalian target of rapamycin (mTOR) induced by epidermal growth factor (EGF) both in vivo and in vitro (Turk et al., 2012). A similar phenomenon was observed in human breast cancer cells, where eicosapentaenoic acid (EPA) and DHA inhibited proliferation and induced apoptosis of MDA-MB-231 human breast cancer cells by changing lipid composition of rafts and decreasing EGF receptor (EGFR) expression in rafts (Schley et al., 2007). In addition, fish oil and DHA have been reported to inhibit DNA binding activity of activator protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) (Liu et al., 2001) which are master transcription factors modulating immune and inflammatory processes. In B cells, it was reported that cross-linking of raft-associated B cell receptors result in cell activation (Allsup et al., 2005). However, if

the antigen binds to B cell receptors that are not located in rafts in immature B cells, the cell will go into apoptosis or anergy (Sproul et al., 2000), indicating that lipid rafts have a great influence on the response and development of B cells.

5.2 Conclusions

In this study, endogenous and exogenous long chain n-3 PUFAs shifted the fatty acid profile in BMMC to a long chain n-3 PUFA-enriched composition with a decreased ratio of n-6 to n-3. Mast cell activation was suppressed by long chain n-3 PUFA inhibition of Lyn and LAT expression and the disruption of FcεRI association with lipid rafts of both resting and activated cells, but not FcεRI expression on cell surfaces or in whole cells. Consistent with reduced degranulation, lipid-derived mediator and cytokine/chemokine production and release, FcεRI-mediated signal transduction was shown to be suppressed by long chain n-3 PUFAs. The IgE/antigen induced phosphorylation of three vital signaling molecules, Lyn, spleen tyrosine kinase (Syk) and LAT, was inhibited. These results support my hypothesis that long chain n-3 PUFAs inhibit mast cell activation by disrupting FcεRI localization and shuttling to lipid rafts and expression of some signaling molecules. The inhibitory effect of long chain n-3 PUFAs on mast cell activation requires cholesterol. Connections among long chain n-3 PUFAs, lipid rafts, receptor signal transduction, and mast cell-associated diseases are indicated.

5.3 Future directions

There are several valuable research questions that can be explored based on the results of this project. First, in addition to influencing the expression of Lyn and LAT,

disrupting FcεRI localization in lipid rafts, inhibiting subsequent signal transduction, suppressing phosphatidylinositol 4, 5-bisphosphate (PIP2) distribution in rafts is another possible mechanism that long chain n-3 PUFAs may disrupt mast cell activation. PIP2 is the precursor of the second messengers, inositol trisphosphate (IP3) and diacylglycerol (DAG), which induce protein kinase C (PKC) activation and calcium response. Inthe present study, long chain n-3 PUFAs were shown to alter lipid composition in lipid rafts or, in other words, raft property. A report has been published showing that lipid raft destabilization can inhibit PIP2 content in rafts (van Rheenen et al., 2005).

Second, the current study raises the question as to how long chain n-3 PUFAs modulate Lyn and LAT in mast cells. PPARs and free fatty acid receptors, GPR40 and GPR120, are possible candidates.

Third, the effects of long chain n-3 PUFAs and/or lipid rafts on signal transduction of other receptors in mast cells could be different than the mechanism described in this study. Mast cells express a broad range of cell surface receptors. The results of chapter 3 have shown that FcεRI is associated with lipid rafts both in resting and activated mast cells, and changes in lipid raft fatty acid profile influenced FcεRI-mediated signal transduction. However, N-methyl-D-aspartate (NMDA) receptor signaling pathway, which is also expressed on mast cells (Daeffler et al., 1999), was not altered by changes in lipid raft property (Nothdurfter et al., 2013). It is worthwhile to study the effect of long chain n-3 PUFAs on signaling pathways of other receptors in mast cells with different properties than FcεRI.

Fourth, the effect of DGLA (20:3 n-6), a n-6 PUFA acting as a precursor for anti-inflammatory 1-series prostaglandins, but not leukotrienes (Levin et al., 2002), is

another candidate that may have protective effects against mast cell activation. As a PUFA, it may also regulate membrane function. Furthermore, the 15-hydroxyl derivative from DGLA inhibits leukotriene synthesis from AA (Samuelsson, 1987). Long chain n-3 PUFAs also function in modulating membrane function and inhibiting eicosanoid production from AA. Thus, a synergistic effect on mast cell activation may exist between DGLA and long chain n-3 PUFAs.

Fifth, based on in vitro results of this study, in vivo studies can be designed to look at the effects of long chain n-3 PUFAs on the whole body. To optimize the effects, it might be necessary to try different combinations of EPA, DHA and DGLA, which may result in an optimized formula for beneficial effects of PUFA supplementation.

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